Molecular Typing of Wine Yeasts: Evaluation of Typing Techniques and Establishment of a database

by

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Summary

The yeast species, *Saccharomyces cerevisiae* and *S. bayanus* are well known for the key role they play during alcoholic fermentation in both wine and beer industries. These yeasts are available in pure active dried form and can be used to produce different wine styles and to manage quality. There are more than 200 commercial wine yeast strains on the market and include naturally isolated strains and hybrids. With all these commercial yeasts available, strain authenticity is very important to the manufacturer of active dried wine yeasts (ADWY) because it can prevent commercial losses and maintain market credibility. It is as important to the winemaker as it may impact wine quality. Various traditional and molecular techniques have been successfully applied to perform quality control of wine yeast strains.

The aims of this study were to evaluate electrophoretic karyotyping (CHEF) and PCR-based methods to distinguish between *Saccharomyces* wine yeast strains and to establish a database containing molecular profiles of commercial strains. CHEF karyotyping was chosen because it is generally used in the wine industry to distinguish between wine yeast strains, but can be time-consuming. Alternatively, PCR-based methods are considered to be reliable and fast. These PCR methods included the evaluation of interdelta regions, multiplex-PCR of mini- and microsatellites, *MET2* gene RFLP analysis and the use of several species-specific primers.

In this study, 62 commercial wine yeast strains, were randomly selected from various manufacturers of ADWY, and two reference strains, *S. bayanus* CBS 380 and *S. cerevisiae* CBS 1171, were evaluated. CHEF karyotyping could successfully differentiate between all 64 yeast strains. The two primer sets used for interdelta amplifications, delta1-2 and delta12-21, yielded 59 and 62 profiles, respectively. Yeast strains considered to be similar or identical according to interdelta amplification results, were resolved with CHEF karyotyping. CHEF karyotyping was proven to be more accurate than interdelta amplifications in distinguishing between commercial wine yeast strains. However, the results of interdelta amplifications were very useful and less time-consuming. The multiplex-PCR of mini- and microsatellite primers only succeeded in identifying a specific band within 55 of the 64 yeast strains including the *S. cerevisiae* reference strain, a possible indication of species specificity. However, oenological designation using *MET2* gene RFLP analysis and species-specific primers indicated that all the commercial strains in this study had a *S. cerevisiae* ancestry. Restriction analysis of the *MET2* gene with *EcoRI* also successfully identified AWRI Fusion and Zymaflore X5 as hybrid yeast strains. A wine yeast database was created and contains three libraries, *i.e.* CHEF karyotypes, delta1-2 and delta12-21 electrophoretic profiles. The database was proven to be functional and showed great accuracy in grouping and identifying test strains. The database has many possible applications, but there is still some optimisation and refinement needed.

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Opsomming

Die *Saccharomyces sensu stricto* kompleks, is bekend vir die belangrike rol wat hierdie giste speel tydens alkoholiese fermentasie in biede wyn en bier industrieë. Dit is om hierdie rede dat kelders rein aktief gedroogte wyngis gebruik vir die produksie van spesifieke wynstyle, asook kwaliteit. Daar is meer as 200 kommersiële wyngiste op die mark beskikbaar en dit sluit natuurlike isolate en hibriede in. Daarom is gisras verifikasie baie belangrik vir die vervaardiger van aktief gedroogde wyngiste asook die wynmaker om finansiële verliese te voorkom en mark vertrouenswaardigheid te handhaaf. Verskeie tradisionele en molekulêre metodes word suksesvol toegepas vir gehalte beheer van die gisrasse.

Die doel van hierdie studie was om elektroforetiese kariotipering (CHEF) en PKR gebaseerde tegnieke se vermoë om tussen *Saccharomyces* wyngiste te onderskei, te ondersoek. Ook deel van die doelwitte was om 'n databasis te skep wat die verskillende elektroforetiese profiele van die kommersiële gisrasse bevat. Tydens hierdie studie is 62 kommersiële gisrasse van verskeie vervaardigers eweka nsig geselekteer. *Saccharomyces bayanus* CBS 380 en *S. cerevisiae* CBS 1171 is as verwysingsrasse gebruik. Elektroforetiese kariotipering (CHEF) is gekies omdat dit een van die mees algemeenste tegnieke is wat gebruik word om tussen wyngiste te onderskei, maar dit word as tydrowend en arbeidsintensief beskou. As 'n alternatief is daar na PKR gebaseerde tegnieke gekyk. Hierdie tegnieke word as betroubaar en vinnig beskou. Verskeie PKR gebaseerde tegnieke is ondersoek, naamlik PKR van interdelta areas, multipleks-PKR van mini- en mikrosatelliete, *MET*2 geen RFLP analise en die gebruik van spesie- spesifieke inleiers. Interdelta amplifikasies en mini- en makrosatelliet inleiers is geselekteer as gevolg van hul vermoë om *Saccharomyces* wyngiste tot op spesie en ras vlak te onderskei. Die *MET*2 geen en spesie-spesifieke inleiers is geselekteer om die kommersiele wyngis as *S. cerevisiae, S. bayanus* of as hibriede te klassifiseer.

CHEF kariotipering kon tussen al 64 giste onderskeid tref. Die twee stelle inleiers wat vir interdelta amplifikasie gebruik was, delta1-2 en delta12-21, het onderskeidelik 59 en 62 profiele gelewer. Gisrasse wat identiese profiele met die delta inleiers gelewer het, kon egter met CHEF kariotipering onderskei word. Die resultate het getoon dat CHEF kariotipering beter tussen die kommersiële wyngiste kon onderskei as die interdelta amplifikasies, maar dat die interdelta amplifikasies nogsteeds goeie onderskeiding toon en dat dit minder tydrowend is. Die multipleks-PKR van mini- en mikrosatelliete kon slegs 'n enkele band in 55 van die 64 giste uitlig. 'n Moontlike aanduiding van spesie spesifiekheid. Die oenologie se groepering volgens *MET2* geen analise en spesies-spesifieke inleiers dui aan dat al die kommersiele wyngiste wat in hierdie studie gebruik is, moontlik van *S. cerevisiae* afkomstig is. Restriksie analise van die *MET*2 geen met *EcoRI* het ook AWRI Fusion en Zymaflore X5 as hibriede geïdentifiseer. Die CHEF kariotipes en interdelta elektroforetiese profiele is gebruik om 'n databasis van die kommersiële *Saccharomyces* wyngiste op te stel. Die databasis is funksioneel en het die toets

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rasse a kkuraat geïdentifiseer en korrek gegro epeer. Die databasis moet egter nog verdere optimisering en verfyning ondergaan.

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This thesis is dedicated to my family and friends for their love, support and inspiration.
Hierdie tesis is aan my familie opgedra vir hulle volgehoue liefde, ondersteuning en inspirasie.

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Biographical sketch

Justin Wallace Hoff was born in George, South Africa on 19 July 1982. He attended Pacaltsdorp Primary School and matriculated at Outeniqua High School in 2000. Justin enrolled at the Stellenbosch University in 2001 and obtained his BSc (Microbial Biotechnology) degree in 2006, majoring in Microbiology and Biotechnology. In 2007, Justin joined the Post-Harvest and Wine Technology Division at ARC Infruitec-Nietvoorbij. He joined the Post graduate Development Programme of the ARC in 2008 and enrolled for a HonsBSc degree in Wine Biotechnology at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 4 (four) chapters. Each chapter is introduced separately.

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Chapter 2 **Literature review**
Molecular typing of *Saccharomyces* wine yeasts: A review of phenotypic and molecular methods

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Molecular typing of wine yeasts: Evaluation of typing techniques and establishment of a database

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Chapter 1

Introduction and project aims

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GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Wine fermentation has been described as a complex microbial process, which involves the interaction of yeasts and bacteria. The conversion of sugars to ethanol can be performed by yeast strains present on grapes (natural microflora) or on winery equipment, a process commonly referred to as spontaneous alcoholic fermentation (Fleet & Heard, 1993). Within the natural flora, the genus *Saccharomyces* is mainly responsible for the domination and completion of alcoholic fermentation (Pretorius, 2000). However, it has become standard practice in the wine industry to use commercially available active dried *Saccharomyces* strains as starter cultures. These strains are derived from natural isolates or from breeding programmes, and sought after phenotypical characteristics include alcohol tolerance (14%), reproducibility and dominance of fermentations, the achievement of low concentration of residual sugars (2-5 g/L), the production of desirable esters and low production of volatile acids, as well as microbial tolerance. The yeast should furthermore minimally impact grape varietal characters (Bisson, 2004; Cocolin *et al*., 2004).

Identification and differentiation of yeast species such as *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* (Naumov *et al*., 2000) and the recently described *S. arboricolus* (Wang & Bai, 2008) is important because of practical implication for the wine, brewery and baking industries. During the last few decades molecular and biological techniques have allowed for characterisation and differentiation of yeasts populations in the vineyard (Degre *et al*., 1989; Pretorius & van der Westhuizen, 1991) and wineries (Hallet *et al*., 1988; Frezier & Dubourdieu, 1992; Pretorius *et al*., 1999; Pretorius, 2000). These molecular techniques have different capacities for taxonomical resolution, and include pulsed field gel electrophoresis (PFGE) (Carle & Olson 1985; Blondin & Vezinhet, 1988; Fernandez-Espinar *et al*., 2001; Oliveira *et al*., 2008), PCR-based procedures ranging from species-specific PCR (Ruy *et al*., 1996; Josepa *et al*., 2000), amplification of intron splice-sites (de Barros *et al*., 1998), amplification of interdelta regions (Ness *et al*.,1993; Legras & Karst, 2003), microsatellite primers (Baleiras Couto *et al*., 1996), PCR-RFLP of rDNA spacer egions (Masneuf *et al*., 1996; Fernandez-Espinar, 2000), restriction analysis of mitochondrial DNA (Fernandez-Espinar, 2001) and AFLP (de Barros *et al*., 1999, Gallego *et al*., 2005). Of these techniques PFGE has the highest resolution for oenological strains, but is seen as time-consuming (Vezinhet *et al*., 1992; Martinez *et al*., 2004).

New wine yeast development forms a major component of the yeast research conducted at ARC Infruitec-Nietvoorbij, The Institute for Wine Biotechnology (IWBT) at Stellenbosch University as well as yeast producers. These yeasts are normally sold by commercial

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companies under licence agreements. Numerous manufacturers/suppliers sell commercial yeast cultures (active dried form) in South Africa, which are either produced locally or abroad. Globally the number of new yeast strains is growing due to ongoing screenings of natural isolated and breeding of strains for improved wine quality and to suite new wine styles dictated by consumer trends. In this regard, strain authenticity is very important, as it can prevent commercial losses and maintain market credibility. As part of quality control after drying, yeasts are normally compared to their respective mother cultures to ensure strain authenticity. Currently, electrophoretic karyotyping (fingerprinting) utilising contour-clamped homogeneous electric field gel electrophoresis (CHEF) is the preferred technology at ARC Infruitec-Nietvoorbij. This technique has proven to be very reliable in accuracy and efficiency for discriminating between various *Saccharomyces* yeast strains (Gomes *et al*., 2000: Tornai-Lehoczki & Dlauchy, 2000). However, as previously mentioned it is time-consuming and costly, and other techniques such as those based on PCR need to be considered, compared and evaluated. It is also obvious that the increasing availability of high-troughput sequencing technologies will play a major role in strain identification in the near future. However, at present this technology is costly and not competitive for routine analysis, and was therefore not considered for this study.

According to the South African Wine Industry Information & Systems (2011), the gross wine production for 2009 and 2010 were 998.6 and 932.7 million litres, respectively. Generally, between 20-30 grams of active dried yeasts (sold as 0.5-1 kg packets) are used to produce a hectolitre of wine. Commercial wine yeast prices may vary from R100-R700 per kilogram and represent therefore a significant market value. Therefore, a constant danger exists that licenced yeasts are being duplicated and produced illegally by competitors. This could mean a loss of possible income in the form of sales, royalties and market share. It also impacts the local wine industry in the sense that intellectual knowledge and competitiveness is lost. Anecdotal evidence has arisen that locally produced yeast may be available in other countries under different names.

1.2 PROJECT AIMS

The main aim of this study is to evaluate various molecular methods to distinguish between commercially available yeast strains and create a database, containing various molecular libraries (CHEF karyotyping the main library), of commercially available wine yeast. The database will be used for comparative studies and to determine genetic relatedness between yeasts.

The specific aims and approaches of this study included:

i. Sourcing 62 commercially available yeast strains from various manufacturers/suppliers
ii. Evaluation of typing techniques;

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a. CHEF karyotyping
b. Amplificaction of Interdelta regions
c. Multiplexing-PCR of mini- and microsatellites

iii. Evaluation of *MET2* gene analysis and species-specific primers as possible oenological designators for commercial yeast strains

iv. Creation of a database containing molecular fingerprint libraries.

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Chapter 2

Literature review

Molecular typing of *Saccharomyces* wine yeasts: A review of phenotypic and molecular methods

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LITERATURE REVIEW

2.1 Introduction

Yeasts are unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results mainly from budding or binary fission. They are characterised by sexual states that are not formed within or on a fruit body (Barnett, 1992). More than 700 species of yeast have been identified (de Barros Lopes *et al*., 1998; Barnett *et al*., 2000).

Yeast strains are associated with the fermentation of food and beverages and are also used in derivations of various food ingredients, which classify these organisms as a processing tool (Fleet, 2006). Wine is a fermented product which is produced either by spontaneous fermentations by the natural microflora present on grapes or winery equipment or by inducing the fermentations with inoculums of actively dried pure cultured yeast strains. Inoculated fermentations increase the likelihood of reliable, rapid and problem-free fermentations. Pure cultures have specific abilities and contribute to the complexity, flavour and quality of the wine (Pretorius, 2000; Vaudano & Garcia-Moruno, 2008). Monitoring of spontaneous or induced fermentations provides an understanding of the dynamics and composition of the total microflora during fermentations and wine environment, and consequently how these organisms affect the wine composition and ultimately the quality (Querol *et al*., 1992; Schutz & Gafner, 1994; Pramateftaki *et al*., 2000). Ecological surveys of wine yeast strains from various areas have been published (Redžepović *et al*., 2002; Fleet, 2003). Population dynamic studies in vineyards have revealed that yeast species are dependent on factors, *i.e* geographical location, climate, grape variety and physical damage to grapes (Khan, 1999; Pretorius *et al*., 1999; Van der Westhuizen, 1999). This has led to the introduction of suitable characterised yeast strains (*Saccharomyces*) for commercial use, which are better adapted to fermentations at higher sugar levels and generally, have a tolerance to ethanol and higher levels of sulphite (Vezinhet *et al*., 1990; Querol & Ramon, 1992; Lavallee *et al*., 1994; Pretorius, 2000).

Currently, more than 200 commercial yeast strains are available globally and mainly consist of natural isolates (diploid, aneuploid or polyploidy) as well as hybrids (Henschke, 2004; Bradbury *et al*., 2005). However, the need for unambiguous identification of wine yeast species and wine yeast strains has always been a prime concern to the wine industry because of economic implications. Past identification has relied on biochemical and physiological properties for characterisation and identification, but can be affected by culturing conditions (Barnett *et al*., 2000, Ribereau-Gayon *et al*., 2006). Molecular approaches to characterisation and identification, have in part, replaced the traditional methods and are based on DNA base composition, genome reassociation, gene sequencing, chromosomal karyotyping and PCR-based methods (Baleiros Couto *et al*., 1995; Esteve-Zarzoso *et al*., 1999; Pretorius *et al*., 1999;

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Pretorius, 2000; Schuller *et al*., 2004; Nisiotou & Gibson, 2005; Pulvirenti *et al*., 2005: Fernandez-Espinar *et al*., 2006).

This literature review discusses taxonomical and ecological aspects of *Saccharomyces* yeasts and focuses on older and new methodology that is used to distinguish between species and strains of this genus.

2.2 Taxonomy

Classification refers to the grouping of organisms in taxa based on their similarities or common ancestral relationships, whereas identification incorporates the idea of comparing unknown organisms to classified species based on similar characteristics (Kurtzman *et al*., 2011). Taxonomy is seen as a collective description of both classification and identification (Barnett *et al*., 2000; Ribéreau-Gayon *et al*., 2006).

Primarily, taxonomist classify yeast species (Ascomycetous, Basidiomycetous) on sexuality or the lack of a sexual phase (Kurtzman, 2003; Ribéreau-Gayon *et al*., 2006) and secondary by the other subdivisions, whereas classification and identification are based on morphological, physiological (nutritional) and molecular criteria (Pretorius *et al*., 1999; Kurtzman *et al*., 2011). Furthermore the molecular taxonomy of yeast is done on grounds of DNA recombination, similarities of DNA base composition, similarities of enzymes, ultrastructure characteristics and cell wall composition (Ribéreau-Gayon *et al*., 2006). The arrangement or grouping of yeast strains into species, species into genera, genera into families, families into orders, orders into classes and classes into divisions conforms to the International Code of Botanical Nomenclature (Greuter *et al*., 1994; Kurtzman *et al*., 2011). The latest version of the code was adopted at the seventeenth international Botanical Congress in Vienna, Austria in 2005 (Kurtzman *et al*., 2011).

2.2.1 Yeast genus*: Saccharomyces*

Currently, taxonomists group yeast into 81 genera and 590 species of which only 19 are considered relevant to wine (Ribéreau-Gayon *et al*., 2006). Meyen in 1883 introduced the genus of *Saccharomyces* and later Hansen (1908) described two species, *Saccharomyces cerevisiae* (beer) and *Saccharomyces ellipsoideus*. During the course of time yeast species were reassigned from and to the *S. cerevisiae* group (Barnett, 1992; de Barros Lopes *et al*., 1998; Pretorius *et al*., 1999). However, it was found that not all yeasts within this group were suitable for wine fermentations (Kurtzman & Fell, 1998).

Progressively a molecular approach divided *Saccharomyces* into genotypically distinct species namely *S. bayanus, S. castellii, S. cerevisiae*, *S. diasensis, S. exiquus, S. kluyveri, S. paradoxus, S. pastorianus, S. servazzii* and *S. unisporus* (Quesada & Cenis, 1995) and later,

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newly defined species such as *S. kunashirensis*, *S. martiniae* (James *et al*., 1997; Kurtzman *et al*., 2010), *S. cariocanus, S. nikatae* and *S. kudriavzevii* (Naumov *et al*., 2000).

Collectively, *S. cerevisiae* and closely related species*, S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* as well as the recently described *S. arboricolus (*Wang & Bai, 2008) are known as the *Saccharomyces sensu stricto complex* (Tornai-Lehoczki *et al*., 1996; Vaughan-Martini & Martini, 1998; Ribéreau-Gayon *et al*., 2006; Kurtzman *et al*., 2011). The yeast species, *S. exiguus, S. castellii, S. servazzii* and *S. unisporus* are known as the *Saccharomyces sensu largo* group, while *S. kluyveri* forms a group on its own. The *Saccharomyces sensu largo* and *S. kluyverii* are also collectively known as the *Saccharomyces lato* group (Kurtzman & Robnett, 2003), which previously also included *S. dairenensis* (Petersen *et al*., 1999).

2.3 Ecological diversity of yeasts: from grape to wine

Wine can be described as a natural product derived from a series of biochemical reactions which are steered by microorganisms such as yeasts. Characteristically, wine environments have low pH values and high sugar levels which limit the growth of microbial species. Yeasts on unripe grapes range from 10 to 10^3 cfu/mL with *Hanseniaspora (Kloeckera*) species usually dominating on the surface of the grapes and representing 50-75% of the total yeast population (Romano *et al*., 2006). Yeast species in lower numbers on unripe to ripe grapes include non-*Saccharomyces* such as, *Rhodotorula, Cryptococcus*, *Candida*, *Brettanomyces*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and a black pigmented yeast-like fungi, *Aureobasidium pullulans* (Romano *et al*., 2006). However, there are increases in the population numbers in freshly extracted grape must from 10^3 to 10^6 cfu/mL as some grapes are already damaged and the yeasts utilise the sugars available. *Saccharomyces* and *Zygosaccharomyces* species also occur on grapes but to a lesser extent (Martini 1993; Fleet *et al*., 2002). *Saccharomyces cerevisiae*, often described as the main wine yeast, does not primarily occur on grapes but is mostly associated with wineries and the equipment used during fermentations. The failure to isolate *S. cerevisiae* from undamaged grapes in laboratories reflects the preference of *S. cerevisiae* for high sugar environments (Martini & Martini, 1990).

During the fermentation process, anaerobic conditions and factors, *i.e* nutrient depletion, antimicrobial activities and the increasing levels of ethanol enlarge selectivity for growth of yeasts, and the numbers of the non-*Saccharomyces* yeasts, *Hanseniaspora* (*Kloeckera*), *Candida*, *Pichia*, *Kluyveromyces* and *Metschnikowia* stagnate at about 10^6-10^7 cfu/mL before decreasing midway through fermentations (Heard & Fleet, 1988, Romano *et al*., 2006). During the later stages of natural wine fermentations the more ethanol tolerant, and therefore more competitive *Saccharomyces sensu stricto* yeast strains, become more predominant (10^7-10^8 cfu/mL) (Romano *et al*., 2006).

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2.4 METHODS FOR IDENTIFICATION AND CLASSIFICATION

2.4.1 Morphological and physiological tests

Morphological investigations of yeast are executed after isolation and growth on selective media. The description of colonies includes, texture, colour, surface, elevation and margin (Kurtzman *et al*., 2011). The morphology description of asexual cells of yeast can also involve observation by microscopy. Furthermore, traditional physiological and biochemical tests include the fermentation of different carbohydrates, growth on specific carbon and nitrogen sources as well as other tests that assess vitamin requirements, splitting of arbutin, acid production from glucose, lipase activity and various others (Kurtzman *et al*., 2011). Several commercial kits are available for the identification of yeast and are based on the physiological traits mentioned above. The first kits produced for oenological yeast were designed by Lafon-Lafourcade & Joyeux, and Cuiner & Leveau in 1979 (Ribéreau-Gayon *et al*., 2006). These tests are conducted on agar plates or in rimless test tubes covered with cotton plugs or sliding caps. Positive or negative results can either be the done by inspecting plates or tubes for growth, formation of gas or the change in pH indicators depending on the test employed (Verweij *et al*., 1999, Kurtzman *et al*., 2011). Automated systems linked to identification software have also made it easier to analyse and conduct these tests. Some of the commercial kits include; API 20C strips (Analytab Products), API ID 32C (BioMerieux), AutoMicrobic (Vitek Systems) and the Auxocolor system (Sanofi Diagnostics Pasteur). Most of these kits generate a seven to ten digit numerical profile that is compared to the database of the supplier or manufacturer to assign a most probable genera or species (Verweij *et al*., 1999, Kurtzman *et al*., 2011).

2.4.2 Fatty acid analysis

Eukaryotic cells are made up of various constituents of whom fatty acids are part. This group of acids include medium chain fatty acids (C8:0, C10:0, C12:0), long chain saturated fatty acids (C14:0, C16:0) and unsaturated fatty acids (C14:1, C16:1, C18:1) (Torija *et al*., 2003). The composition of fatty acids im membranes differs from species to species. Fatty acids are extracted by means of saponification focussing on methyl esters as they are volatile, followed by gas-liquid chromatography. Through the decades cellular fatty acid analysis has been used to discriminate between different yeast species, including wine strains (Khan, 1999; van der Westhuizen, 1999). Fatty acid analysis was performed by determining the mean relative percentages of cellular fatty acids (Tredoux *et al*., 1987; Augustyn, 1989; Augustyn & Kock, 1989). It was possible to separate spoilage yeast from grape yeast by means based on the presence or absence of linoleic or linolenic acids (Augustyn & Kock, 1989). However, Torija *et al*. (2003) showed that growth media and environmental conditions can affect the composition of yeast cell membranes. This technique has been described as being reliable, but time

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consuming. Another disadvantage is that fatty acid analysis can not always be applied to yeast strains with rigid cell membranes.

2.4.3 Electrophoretic karyotyping

Electrophoretic karyotyping has been used widely over the last few decades. This technique is based on the electrophoretic separation of intact, undigested chromosomal DNA molecules (Lai *et al*., 1989). Generally, electrophoretic karyotyping is comparable with genome macro restriction patterns obtained by genome digestion with low frequency restriction endonucleases which are separated by agarose gels (Shin *et al*., 2004; Chen *et al*., 2005). The preparation of full-length chromosomal DNA includes growing yeasts in liquid media and subjecting the cells to *in situ* lyses processes till immobilised on gels (Carle & Olson, 1985). This results in the elimination or addition of chromosomal DNA and can be used in the identification of polymorphisms in homologous chromosomes within the genome (Wolfe & Shields, 1997; Casaregola *et al*., 1998; Keogh *et al*., 1998). Once prepared, the DNA is separated using a technique commonly referred to as pulsed field gel electrophoresis (PFGE). This technique uses variations in various parameters including the variation of time intervals and in the force of the electrical field, agarose concentration, temperature and the orientation or the gradient of the field. There are many variations of PFGE available for the separation of chromosomal DNA, including contour-clamped homogenous electric field (CHEF), field inversion gel electrophoresis (FIGE), orthonogal field alternation gel electrophoresis (OFAGE) and transverse alternating field electrophoresis (TAFE). CHEF focuses on the transverse angle reorientation and maintains homogeneous electrical field in combination with a horizontal gel. During CHEF the direction of the electric field is changed electronically to reorientate the DNA which is done by changing the polarity of an electrode array.

The use of electrophoretic karyotyping has led to a better understanding of the organisation as well as the characterisation of eukaryote genomes. The technique has shown high efficiency and accuracy in discriminating between yeasts of the *Saccharomyces sensu stricto* group as well as intraspecifically between *S. cerevisiae* strains, especially in the wine industry, where discrimination of commercial strains is very important (Degre *et al*., 1989; Nadal *et al*., 1996; Fernandez-Espinar *et al*., 2001; Cocolin *et al*., 2004; Pulvirenti *et al*., 2005; Le Jeune *et al*., 2007). Using this technique combined with principle component analysis (PCA) Cardanali & Martini (1994) showed that yeast strains of the *Saccharomyces sensu stricto* groups cluster together. However, this can only be done when looking at the presence of specific chromosomes in the yeast karyotypes.This technique have also been used in population dynamic studies of *S. cerevisiae* (Longo & Vezinhet, 1993; Schutz & Gafner, 1994; Van der Westhuizen *et al*., 1999). Even with newer technologies coming to the fore ground, electrophoretic karyotyping still shows greater resolution than some other techniques, which include randomly amplified polymorphic DNA (RAPD) and microsatellites or other genetic

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markers for *S. cerevisiae* (Oliveira *et al*., 2008). Disadvantages of electrophoretic karyotyping include, being time-consuming, laborious and relatively expensive.

2.4.4 Restriction analysis of *mt*DNA

Mitochondrial DNA (*mt*DNA) of *S. cerevisiae* is small molecules between 65-80 kb in length and show high variability when subjected to restriction, which make it very polymorphic (Fernandez-Espinar *et al*., 2006). *Mt*DNA are rich in A, T and in part G and C, and it is the GC content difference, between nuclear and *mt*DNA that can be exploited by total fungal DNA digests (Fernandez-Espinar *et al*., 2006). Isolation of *mt*DNA can be very difficult and can become very laborious. Specific methods for the isolation of *mt*DNA were developed by Querol *et al*. (1990) and Lopes *et al*. (2002), with the latter being preferred as *mt*DNA can be analysed without previous isolation and purification requirements. When nuclear DNA is digested, a number of smaller fragments are noticeable, but cannot be detected by normal agarose gel electrophoresis. However, the *mt*DNA will be superimposed on the shadow of the nuclear DNA (Fernandez-Espinar *et al*., 2006). Once the *mt*DNA has been isolated, it can be digested with restriction enzymes (e.g. *HinfI, Hae III & RsaI*) and the restriction patterns can be analysed by agarose gel electrophoresis (Guillamon *et al*., 1994; Fernandez-Espinar *et al*., 2001). This technique can be used to characterise and identify reference and commercial wine strains (Vezinhet *et al*., 1990; Querol *et al*., 1992; Guillamon *et al*., 1996; Nadal *et al*., 1996; Fernandez-Espinar *et al*., 2001; Esteve-Zarzoso *et al*., 2004; Martinez *et al*., 2004; Schuller *et al*., 2004). This technique can also be used to do population dynamic studies during fermentations (Araujo *et al*., 2007).

2.4.5 Fourier-transform near infrared (FTIR) spectroscopy

Fourier-transform near infrared (FTIR) spectroscopy was developed in 1960 and has been used in various routine and research applications. The basic principle of FTIR is infrared light that is directed through an internal reflection element with a high refraction index. The infrared beam is then reflected off the back of the sample surface (tissue, cell smears, etc.). If the samples have lower refraction indexes, a total internal reflection is obtained (Wenning *et al*., 2002). The depths of penetration rely on several parameters and primarily include the refraction of the object. Radiation of spectroscopy is divided into near, middle and far infrared. The relative success of this method is directly dependent on the complexity within a reference spectral library (Kummerle *et al*., 1998).

Fourier-transform near infrared analysis does not require extensive sample preparations and can be used for various applications, including cell wall structural analysis (Gonzalez-Ramos & Gonzalez, 2006) and differentiation of *S. cerevisiae* strains (Kummerle *et al*., 1998; Galichet *et al*., 2001; Wenning *et al*., 2002). The cell wall of *S. cerevisiae* makes up 15-30% of the dry weight of cells and 25-50% of the volume (Galichet *et al*., 2001). The cell wall is

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composed mainly of mannoproteins and it is these attributes that are used in the identification of yeasts. Osborne (2007) used attenuated total reflectance (ATR)-FTIR to discriminate between yeast phenotypes. The author used different forms of yeast cultures; i.e actively dried yeast, powder and pellets, and obtained suitable chemical fingerprints for identification of yeast strains. FTIR spectroscopy has shown great discriminatory characteristics in differentiation of yeast strains (Cozzolino *et al*., 2006, Osborne, 2007). This technique is fast and a relatively simple method for finding differences between yeast strains, grape cultivars and also different wines (Osborne, 2007). Combining of FTIR spectroscopy with mathematics and chemometrics (Esbensen, 2002) expands the capabilities of this technique in looking for correlations between strains as well as their environment (Osborne, 2007). Disadvantages of FTIR spectroscopy include the acquisition of expensive equipment and difficulties interpreting spectral results.

2.4.6 MALDI-TOF mass spectroscopy

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was introduced by Karas & Hillenkamp (1988). During the process the sample is embedded in a crystalline structure of small organic compounds (matrix) and is deposited on a conductive support for irradiation with a nanosecond laser. The energy from the laser causes structural decomposition of the irradiated crystal and generates particle clouds from which ions are extracted by an electrical field. These ions accelerate through this field and eventually reach a detector where masses are calculated by their time of flight (TOF), resulting in a spectrum being obtained. The masses are in a numerical data format and can be used for direct processing and analysis (Jurinke *et al*., 2004).

MALDI-TOF mass spectrometry (MS) relies on the production, separation and detection of gas-phase ions (Jurinke *et al*., 2004). In the past thermal vaporization methods were used to transfer molecules into a gaseous phase. Ionization methods include electron impact (EI) and chemical ionization (CI). However, during above mentioned methods biomolecules undergo decomposition and fragmentation, which could have a negative impact. If this is taken into consideration, nucleic acid analysis has been limited to molecules the size of dinucleotides (Takeda *et al*., 1991). The development of plasma desorption (PD) methods made oligonucleotide analysis with a mass range of up to 3000 Da (10 nucleotides) possible (Viari *et al*., 1988). Mass spectrometric tools were not widely used for routine applications in the biological sciences until the discovery of electrospray ionization mass spectroscopy (ESI-MS) and MALDI-MS (Jurinke *et al*., 2004).

MALDI-TOF MS is considered to be rapid, reliable and cost effective (but for the instrumentation). This technique can be used for qualitative DNA analysis which include, single nucleotide polymorphism (SNP) analysis (Little *et al*., 1997), microsatellite analysis (Braun *et al*., 1997), DNA sequencing (Koster *et al*., 1996, Kirpekar *et al*., 1998), and quantitative analysis such as allele frequency determination and gene-expression analysis (Ross *et al*., 2000,

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Buetow *et al*., 2001, Ding & Cantor, 2003). Recently MALDI-TOF MS was used for the identification of bacteria (Bizzini *et al*., 2010), mycobacteria (Pignone *et al*., 2006) and fungi (Marklein *et al*., 2009; Stevenson *et al*., 2010). During these studies this technique had a high resolution at genus and species levels.

2.4.7 PCR-BASED TECHNIQUES

2.4.7.1 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) is a molecular *in vitro* technique that is widely used for the amplification of specific DNA regions, which lie between two areas of a known sequence. It was invented by Kary Mullis in 1983 and made use of Klenow fragments (*Eschericia coli*) DNA *pol I*. This original technique was simplified so that single or double stranded DNA could be used as the template (McPherson & Moller, 2006). Oligonucleotide primers are short single stranded DNA molecules. When amplification take place the primers bind to complementary sequences on the DNA sample which has been denatured (McPherson & Moller, 2006). These amplified fragments can then be separated and visualised in agarose gels. The technique is seen as a tool for detection and characterisation. PCR is characterized by its rapidity, sensitivity, robustness and reproducibility.

2.4.7.2 Randomly amplified polymorphic DNA (RAPD)

This analytical DNA marker system was introduced by Welsh & McClelland (1990) and Williams *et al*. (1990). This PCR based technique makes use of arbitrary primer(s), which with characteristically low hybridization temperature amplifies a variety of different size bands along the whole genome. Quick fingerprinting profiles are obtained, which in turn, can be used for analysis of yeast genetic relatedness or relationships (Fernandez-Espinar *et al*., 2006). The banding patterns from RAPDs are usually better visualised when immobilized on polyacrylamide gels (Stift *et al*., 2003). The remarkably distinctive banding patterns of the amplified products can be used for the identification or characterisation of species and different strains within species (Bruns *et al*., 1991; Baleiras Couto *et al*., 1995; Paffetti *et al*., 1995; Oliveira *et al*., 2008). Gallego *et al*. (2005) compared this technique to the amplified fragment length polymorphisms (AFLPs) and microsatellites to discriminate between various wine yeast strains and obtained similar results. Typically described as being a fast and a straight-forward technique whereby low amounts of genetic material, or no previous knowledge of DNA sequences, are needed. In general a major disadvantage of the technique is, however, a low reproducibility, which may be due to the use of low hybridisation temperatures.

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2.4.7.3 Interdelta regions

Delta elements are approximately 300 bp in length and are the flanking regions of the retrotransposons TY1 and TY2 (Cameron *et al*., 1979; Krastanova *et al*., 2005)). They can also be found apart from these retrotransposons and are then referred to as solo delta elements (Lavallee *et al*., 1994). These flanking regions are also found adjacent to the transfer RNA genes (Eigel & Feldman, 1982). About 300 such elements are found in the genome of S288c, a *S. cerevisiae* laboratory strain, and this makes them excellent targets for polymorphisms (Lavallee *et al*., 1994).

Delta primers 1 and 2 have been used to analyse intraspecific variability and to distinguish between various *S. cerevisiae* strains (Ness *et al*., 1993). The results were comparable with results obtained from mitochondrial DNA restriction analysis and electrophoretic karyotyping of chromosomal DNA (Fernandez-Espinar *et al*., 2006). The later design and application of delta primers 12 and 21 in combination with primers 1 and 2, respectively, yielded better polymorphic banding patterns as illustrated in Fig. 2.1 (Legras & Karst, 2003). Schuller *et al*. (2004) gave more credibility to delta primers when they identified twice as many strains as Ness *et al*. (1993) in a similar study. Current trends in technology also allow sequencing of these interdelta markers followed by analysis with capillary electrophoresis (Tristezza *et al*., 2009). Advantages include the ease of use and reduced time-consumption. However, disadvantages include problems regarding DNA concentration, as optimal DNA concentrations are needed for reproducibility and the appearance of ghost banding patterns during analysis due to the low annealing temperatures.

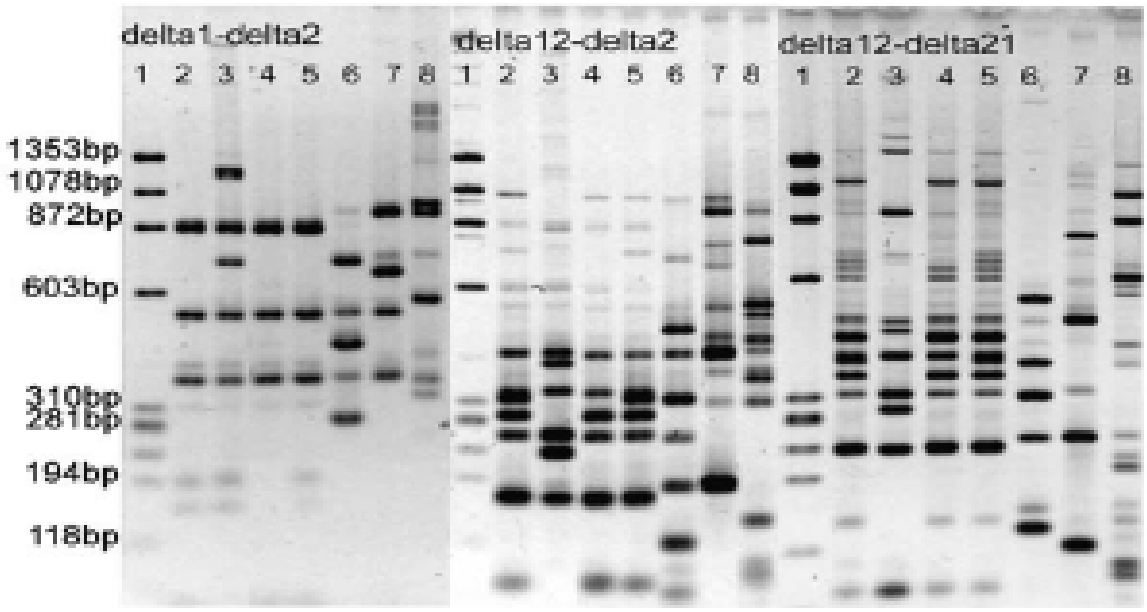

Figure 2.1

Polymorphic banding patterns using various primers for interdelta regions to distinguish between strains of *Saccharomyces* yeasts (Adapted from Legras & Karst, (2003).

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2.4.7.4 Microsatellite analysis

Microsatellite analysis is the genetic tagging by synthesized oligonucleotides complementary to single repetitive sequences, present in the genome of the organisms. These repetitive sequences are generally referred to as microsatellites (Fernandez-Espinar *et al*., 2006). In yeasts these regions may vary from 200 to 3500 bp in length, which can be sufficiently visualised by agarose as well as polyacrylamide gels (Fernandez-Espinar *et al*., 2006). Frequently used satellites include GTC_5, GTG_5, $GACA_4$, GAG_5 and the M13 bacteriophage sequence (Fig. 2.2). This technique is a prime example of single primer (oligonucleotide) amplified reactions (SPAR) (Britos dos Santos *et al*., 2007). This technique differs from RAPDs in that it utilises a higher annealing temperature of 55°C instead of 37°C, which enhances specific oligonucleotide hybridisation and coincides with a higher resolution and reproducibility (Stephan *et al*., 2002; Dalle *et al*., 2003; Fernandez-Espinar *et al*., 2006). These techniques have been useful in the identification of *Saccharomyces cerevisiae* strains (Baleiras-Couto *et al*., 1996; Gonzalez Techera *et al*., 2001; Hennequin *et al*., 2001).

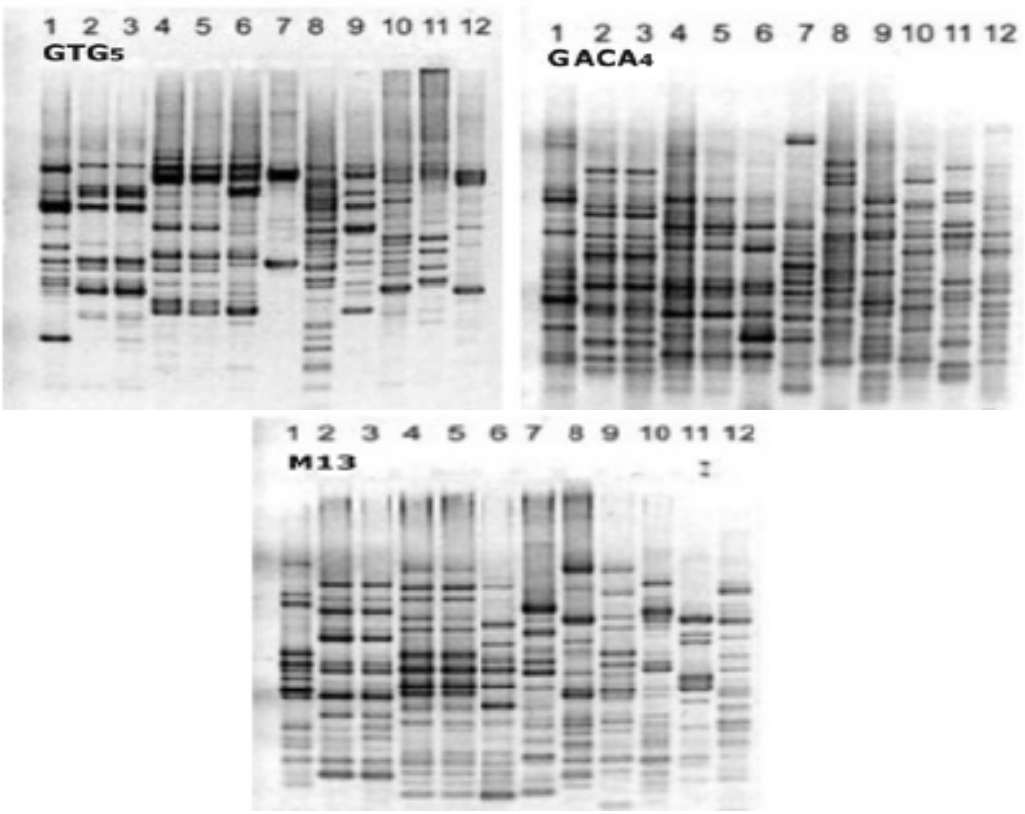

Figure 2.2

An illustration of the fingerprinting capabilities of micro- and minisatellites (Adapted from Brito dos Santos *et al*., 2007).

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2.4.7.4.1 Microsatellite loci

The utilisation of microsatellites also includes the study of microsatellite loci that are scattered throughout the genome of an organism, which is made possible by whole genome sequencing (Bradbury *et al*., 2005). In this case the complete sequence of *S. cerevisiae* genome allows for the identification of these regions as the absolute sizes of the microsatellite markers are known. Some of the most frequently utilized loci include YOR267C, SC8132X, SCPTSY7 (Techera *et al*., 2001); ScAAT1-ScAAT6 (Schuller *et al*., 2004); YML091C, YOL109 W, YFR028C, YPL009C, YDR160 W, YLL049, YBR240C, YGL014 W and YGL139 (Richards *et al*., 2009). These loci can also be used for multiple samples and multiplex-PCR reactions where two or more loci are amplified (Vaudano & Garcia-Moruno, 2008; Richards *et al*., 2009). Results are expressed as a number of repeats of the loci. These loci have been identified and used in studies to successfully discriminate between *S. cerevisiae* strains (Field & Willis, 1998; Perez *et al*., 2001; Techera *et al*., 2001; Malgoire *et al*., 2005) and evaluated to distinguish between commercially available yeast strains (Schuller *et al*., 2004; Bradbury *et al*., 2005; Legras *et al*., 2005; Vaudano & Garcia-Moruno, 2008). This technique has the same discriminatory resolution as interdelta regions, but less than electrophoretic karyotyping. The advantages of this technique are the transferability between organisms and computer translations are effortless, and highly reproducible.

2.4.7.5 Restriction fragment length polymorphism (RFLP)

This technique highlights possible differences in largely homologous DNA sequences and can be detected by the presence of fragments of different lengths generated by the digestion with restriction endonucleases. Fragments can be hybridized by using probes and for characterisation of specific genotypes at a specific locus. These probes are usually short single low copies of genomic DNA or cDNA and are specifically chosen to detect moderate to high polymorphisms in the fragments. As with most molecular techniques the application helps with the genetic variation and helps with genetic mapping (Fernandez-Espinar *et al*., 2006). Advantages of this technique include a high resolution between species. The disadvantage of this technique is that it can laborious. Prime examples of RFLPs are *MET2* gene analysis and ribotyping.

2.4.7.5.1 *MET2* gene RFLP analysis

MET2 gene analysis is based on the principle of RFLP. Hansen & Kielland-Brandt (1994) used *MET2* gene RFLP analysis for the delimitation of wine yeasts, *S. cerevisiae* and *S. bayanus*. Commercial wine yeasts are generally classified either *S. cerevisiae* or *S. bayanus*, albeit incorrectly, depending on their ability to ferment galactose (Ribéreau-Gayon *et al*., 2006). The *MET2* gene codes for synthesis of homoserine acetyltransferase and these DNA sequences of this gene differ for these two species. Complimentary oligonucleotides amplify a part of the

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gene located on the outer flanks whereby a ~600 bp (580 bp fragment, Masneuf *et al*., 1998) amplicon was obtained. Restriction endonucleases, *EcoRI* and *PstI*, were used to cleave the *MET2* gene amplicon of *S. cerevisiae* and *S. bayanus*, respectively (Masneuf *et al*., 1998). In the case of *EcoRI*, two fragments (369 bp, 211 bp) were obtained when the *MET2* gene product of *S. cerevisiae* was cleaved. For *S. bayanus* no cut fragments were visible. For *PstI* the reverse effect was observed, whereby two fragments (365 bp, 215 bp) for *S. bayanus* was visible and no fragment was visualized in *S. cerevisiae.* This PCR-RFLP analysis of the *MET2 gene* also proved useful in demonstrating the existence of natural hybrids within the *Saccharomyces sensu stricto* complex (Masneuf *et al*., 1998).

2.4.7.5.2 *Ribotyping*

Ribotyping is another area where RFLP has been useful and refers to the amplification of ribosomal genes. These areas or regions would include the 5.8S, 18S and 26S (Fig. 2.3) ribosomal genes which are grouped in tandem to form transcription units. These transcription units are repeated between 100-200 times in the genome. Other regions include the internal transcribed spacer (ITS) and external transcribed spacers (ETS), which are areas that are transcribed, but not processed. The transcription units are also separated by intergenic spacers called (IGS). These ribosomal regions have become the tools for identifying phylogenetic relationships between all living organisms (Kurtzman *et al*., 2011) and between yeasts (Kurtzman & Robnett, 1998). According to Li (1997) the transcribed units are more likely to be similar for strains of the same species than for different species. In general the specific regions on the subunits commonly referred to as domain D1/D2, on the 18S (James *et al*., 1997) and 26S (Kurtzman & Robnett, 1998) have been sequenced.

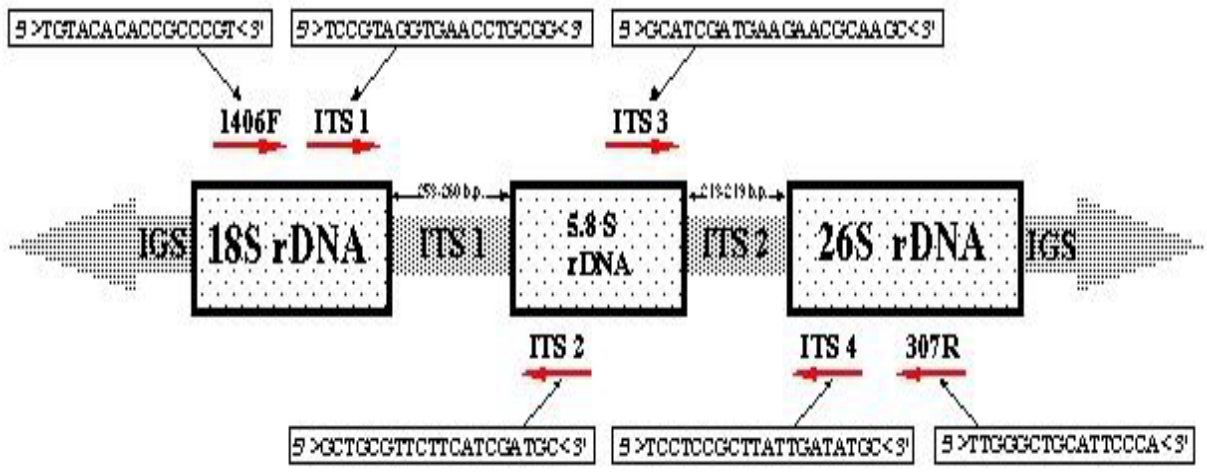

Figure 2.3

Illustration of the structure of nuclear ribosomal DNA.

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According to Kurtzman & Robnett (1998) when assigning unknown yeast or yeast strains to a specific species, the nucleotide sequences in these regions can be used to measure homology to known or related yeasts. Furthermore, the amplification and restriction profiling of these regions and the use of fluorescent dyes have yielded notable results in identifying more strains within specific species (Kurtzman & Robnett, 1998). Dlauchy *et al*. (1999) used specific primers NS1 and ITS1 to amplify regions of the 18S gene, which was then digested with enzymes (*AluI*, *HaeIII*, *MspI* and *RsaI*). White *et al*. (1990) used primers ITS1 and ITS4 (Fig. 2.3) to amplify regions of the 5.8S gene, which was also extensively used for the identification of yeast strains in wine or related industries with relative success (Guillamon *et al*., 1998; Esteve-Zarzoso *et al*., 1999; Fernandez-Espinar *et al*., 2000; de Llanos *et al*., 2004). This technique has also been useful in the studies of reference strains (Ramos *et al*., 1998; Fernandez-Espinar *et al*., 2000; Cadez *et al*., 2002; Esteve-Zarzoso *et al*., 2003; Naumova *et al*., 2003). The non-transcribed areas, 18S gene, ITS region and 26S gene have been widely used by various authors to identify species in the *Saccharomyces sensu stricto* group (Baleiras-Couto *et al*., 1996; Smole-Mozina *et al*., 1997; Tornai-Lehoczki & Dlauchy, 2000; Caruso *et al*., 2002 Capece *et al*., 2003; Vasdinyei & Deak, 2003; Fernandez-Espinar *et al*., 2006). The internal transcribed regions (ITS) has also been targeted by restriction analysis with *DraI* and *HaeIII* to identify and characterize yeast populations with oenological significance, as well as species in the larger *Saccharomyces sensu stricto* group (Esteve-Zarzoso *et al*., 1999; Granchi *et al*., 1999; Redzepovic *et al*., 2002; dos Santos *et al*., 2007).

In recent years amplified ribosomal rDNA restriction analysis (ARDRA) has been developed with the focus on the 16S/18S rDNA. Amplification of this region is followed by either the use of one restriction enzyme or the sequential usage of several in this case *MseI*, *BfaI* and *AluI* (Rodas *et al*., 2003). ARDRA has shown great resolution for discriminating between LAB bacteria, which include the likes of *Lactobacillus brevis*, *Lactobacillus paracasei*, *Oenococcus oeni*, as well as a few others (Rodas *et al*., 2003). ARDRA is helpful in detecting spoilage microbiota in wine, which include spoilage LAB and yeasts (Fröhlich *et al*., 2009).

2.4.7.6 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a hybrid technique of RFLP and RAPD. Firstly, genomic DNA is digested by means of restriction enzymes, usually two different ones, and these fragments are then amplified. The primers are seen as adapters that ligate to restriction enzyme sites and will only amplify subsets of the fragments (Vos *et al*., 1995). During separation larger to smaller banding patterns are observed, which suggests mono- to polymorphisms. This technique is laborious and expensive. Advantages include the high discriminatory power and good reproducibility, especially when applied to detect or determine genetic variation within *S. cerevisiae* strains (Gallego *et al*., 2005). Various other authors investigated the use of AFLPs for identifying intraspecific differences of *S. cerevisiae* strains (de

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Barros Lopes *et al*., 1999; Caruso *et al*., 2002; Esteve-Zarzoso *et al*., 2010). This technique has also been useful in genetic mapping and evolutionary studies whereby large units of loci distributed along the genome have been identified (Gallego *et al*., 2005).

2.4.7.7 Real-time PCR/Quantitative PCR

This specific PCR based technique was developed in 1996 (Wilhelm & Pingoud, 2003). During the PCR process (cycle after cycle) the amplified products can be monitored. This type of quantification and detection is done via fluorescent signals, which increase in every cycle. To visualize the signal a thermocycler with a detection system is required to capture and quantify the amplification process (Fernandez-Espinar *et al*., 2006).

Fluorescence of products is generated through the binding of agents or probes (SYBR Green). The probes can be divided into three groups, namely hydrolysis probes- (Taqman), loop-shaped- (with inverted repeats regions (ITR)) and hybridization probes (fluorophores). The most commonly used are the hydrolysis probes (Taqman probes). Fluorescence occurs when a donor photochrome binds to an acceptor photochrome. The signal becomes dependant on whether both photochromes are attached. The signal becomes visible when the exonuclease property of Taq polymerase activates the donor photochrome of the rest of the probe, hence binding to the sequence of interest (Wong & Medrano, 2005; Querol & Fleet, 2006). Compared to classic PCR primers, the real-time probes show greater specificity. Post process analysis is not needed as data is computer generated as illustrated in Fig. 2.4. Advantages of the technique include high specificity, sensitivity and quantification takes less time compared to common PCR. Disadvantages include the forming of dimers and non-specific products through amplification resulting in an over estimation of the DNA concentration. Variuos publications review the important aspect of real-time PCR in full (Wong & Medrano, 2005; Fernandez-Espinar *et al*., 2006).

This technique is used in the wine and yogurt industries for the detection of spoilage organisms. Real-time PCR is also useful in the study of phylogenetic relationships among yeast species. Applications of real-time PCR include: quantification of gene expression, array verification, DNA damage measurement, quality control or assay validation, pathogen detection and genotyping (Fernandez-Espinar *et al*., 2006). Numerous authors have used quantitive PCR (QPCR) in rapid identification and enumeration of *S. cerevisiae* and differentiating strains of the *Saccharomyces sensu stricto* group (Martorell *et al*., 2005; Hierro *et al*., 2006; Salinas *et al*., 2009). Current application of real-time PCR includes the investigation of protein haze formation in wine (Gonzalez-Ramon *et al*., 2006).

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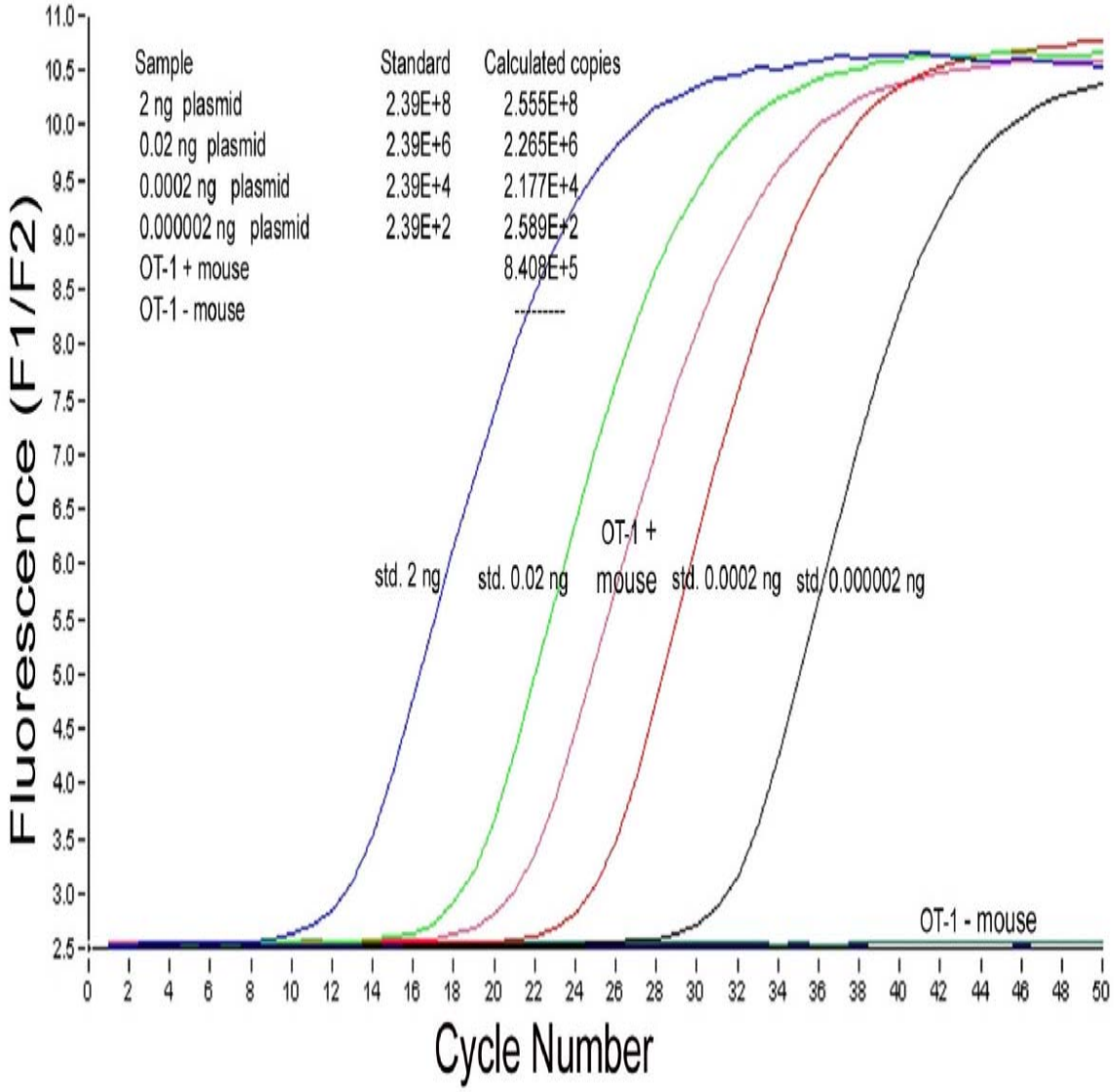

Figure 2.4

A typical spectrum of the amplification process after real-time PCR.

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2.4.7.8 PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

This PCR based technique was first introduced to microbial environmental science by Muyzer *et al*. (1993). PCR-denaturing gradient gel electrophoresis (PCR-DGGE) is based on the separation of same length DNA fragments, but of different sequences. The decreasing electrophoretic mobility of partially melted double-stranded (dsDNA), the concentration at which DNA dissociates, affects the mobility through mediums (agarose or polyacrylamide gels). The complete denaturation of fragments is prevented through GC (guanine and cytosine) clamps attached to the primers). Muyzer *et al*. (1993) used PCR-DGGE to differentiate rRNA genes. Various authors have used the reliability of this technique to distinguishing between yeast strains present in wine fermentations to great effect (Cocolin *et al*., 2000; Mills *et al*., 2002; Nielsen *et al*., 2005). This technique is highly effective when screening for *S. cerevisiae* during the fermentation process and was used to show that *S. cerevisiae* was present during both alcoholic and malolactic fermentation (Renouf *et al*., 2007; Sieberitz, 2007). This was also confirmed with the conventional plating on growth media.

2.4.7.9 Temporal temperature gel electrophoresis (PCR-TTGE)

Another technique similar to PCR-DGGE is PCR-temporal temperature gel electrophoresis (PCR-TTGE), which is based on the linear temperature gradient separation of DNA molecules and as with DGGE; molecules are separated on gels due to diverse sequence mobility. PCR-TTGE has been used for the genetic characterisation of commercially dried yeast and isolated strains in the brewery industry (Gonzalez *et al*., 2001; Giusto *et al*., 2006). This technique is based on PCR and is not too time consuming or drawn out. Results are obtained fast and analysed quickly.

2.4.7.10 Nested specifically amplified polymorphisms (nSAPD-PCR)

This adaptable and versatile technique was developed for strains and genotypes and determination of various organisms, which include LAB bacteria (*Oenococcus oeni, Pediococcus parvulus* & *Lactobacillus hilgardii)* and yeasts (*Saccharomyces cerevisiae*, *Dekkera bruxellensis* and *Candida* species) (Fröhlich & Pfannebecker, 2007). This technique is based on RAPD-PCR and the usage of specific oligonucleotides including a *NotI* recognition site. The process includes two PCR reactions and a set of 20 oligonucleotides. The first reaction utilizes 4 oligonucleotides to amplify the DNA of interest and 16 for the second reaction, which binding sites are nested within the products of the first set of primers as illustrated in Figure 2.5 (Fröhlich & Pfannebecker, 2007). These reactions can be performed in the presence of an enhancer solution for specificity. The primer sets used during nested specifically amplified polymorphisms -PCR (nSAPD-PCR) are also not restricted to small groups of species, in contrast to that used during RAPD-PCR. This technique has high resolution at species and strain level and is characterized by high levels of reproducibility (Fröhlich *et al*., 2009). Specific

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nSAPD-PCR reactions have been designed for *Brettanomyces* and *Dekkera* strains in sherry (Ibeas *et al*., 1996).

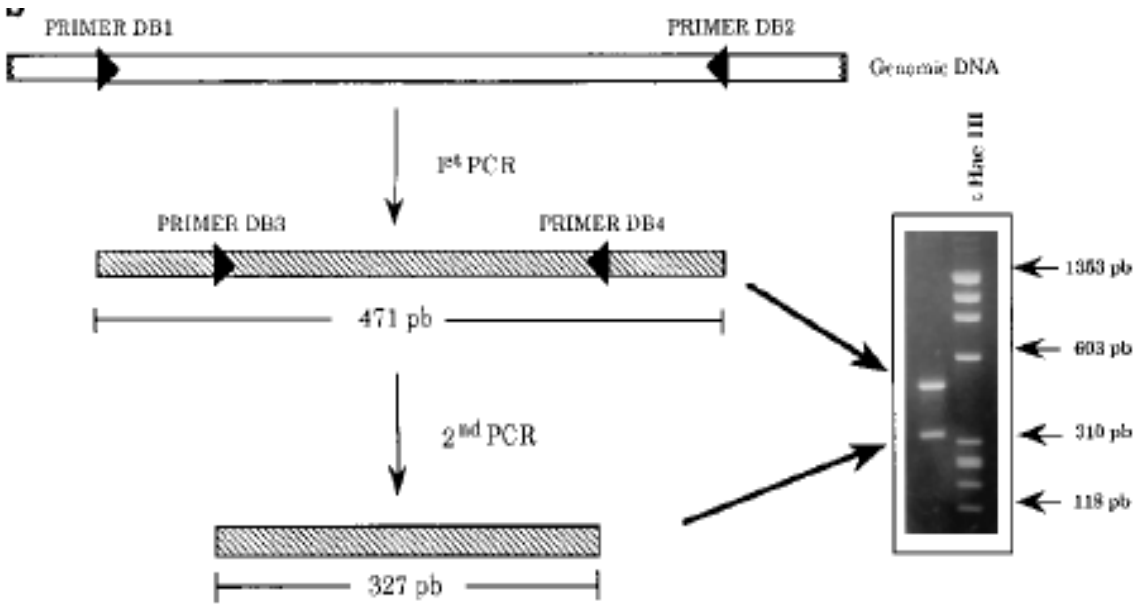

Figure 2.5

An illustration of a typical Nested PCR method**.** (Adapted from Ibeas *et al*., 1996)

2.4.7.11 Enterobacterial repetitive intergenic consensus elements (ERIC) - and repetitive extagenic palindromic elements (REP)-PCR

Two PCR methods, enterobacterial repetitive intergenic consensus elements (ERIC) - and repetitive extagenic palindromic elements (REP)-PCR, are such explorations and have yielded interesting results in the characterisation and identification of yeast strains. ERIC and REP have been used to identify bacterial strains and species in the past (Sharples & Lloyd, 1990; Hulton *et al*., 1991; Versalovic *et al*., 1991). Yeasts studies utilising these PCR methods proved useful in characterising 15 different species and strains in grape must and wine (Hierro *et al*., 2004). Table 2.1 and 2.2 indicate fragments obtained for reference yeast strains from the CECT collection. Hierro *et al*. (2004) found REP-PCR to be inadequate for intraspecific characterisation of yeast strains but useful for identification of yeasts. These PCR techniques can be used to differentiate between *S. cerevisiae* and *S. bayanus* (Hierro *et al*., 2004). These two techniques are rapid and reliable, but there are concerns with reproducibility as nonspecific amplification can be obtained. These techniques are an inexpensive way for winemakers and researchers to identify oenological relevant yeast species.

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Table 2.1 Size fragments obtained using ERIC-PCR for yeast collection strains by Hierro *et al*. (2004).

Species	**CECT Designation**	**Fragment**
Sacharomycodes ludwigii	1382, 1371	825, 925, 1050
Schiz. Pombe	1378, 1379, 10685	470, 850, 1200, 1400
T. deldrueckii	1880, 10558, 10676	180, 330, 420, 500, 580, 620, 850, 1250, 1400
Z. bailii	11041, 11042, 11043	150, 200, 380, 500, 620, 850, 950, 980, 1250
Z. rouxi	1230, 1232	350, 650, 850, 1400, 1500
S. bayanus	1941, 1969	220, 520, 600, 650, 820, 980, 1150, 1200, 1500
S. cerevisiae	1171, 1942	220, 270, 320, 590, 630, 800, 980, 1200, 1250, 1400

Table 2.2 Size fragments obtained using REP-PCR for yeast collection strains by Hierro *et al.* (2004).

Species	**CECT Designation**	**Fragment**
Sacharomycodes ludwigii	1382, 1371	560, 600, 710, 1350
Schiz. Pombe	1378, 1379, 10685	200, 520, 650, 710, 830, 900, 1150, 1250, 1750
T. deldrueckii	1880, 10558, 10676	320, 400, 550, 590, 510, 650, 730, 830, 1000, 1050, 1200, 1500
Z. bailii	11041, 11042, 11043	180, 520, 590, 600, 780, 850, 930, 1000, 1350
Z. rouxi	1230, 1232	320, 380, 470, 540, 700, 790, 930, 1000, 1050, 1250
S. bayanus	1941, 1969	160, 350, 420, 530, 900, 1000, 1100, 1300
S. cerevisiae	1171, 1942	150, 340, 570, 700, 900, 1000, 1080, 1100, 1250, 1300

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2.4.7.12 Single strand conformation polymorphism PCR (PCR-SSCP)

Single strand conformation polymorphism PCR is an electrophoretic separation technique, which separates single stranded nucleic acids based on subtle differences in sequence. This results in different secondary structures being formed and causes a measurable difference in mobility through a gel. SSCPs are allelic variants of inherited, genetic traits and can be used as markers. Analysis can detect DNA polymorphisms and mutations at multiple places in fragments (Orita *et al*., 1989) This technique was applied for the first time to study point mutations in humans (Orita *et al*., 1989) and a number of human and plant-pathogenic fungi (Walsh *et al*., 1995; Kumeda & Asoa, 1996; Kong *et al*., 2003; Wang *et al*., 2008). This technique discriminates between a large number of Ascomycete and Basidiomycete yeast including *S. cerevisiae* (Wang *et al*., 2008), as well as being an excellent detector for gene mutations and variation analysis (Dockhorn-Dworniczak *et al*., 1991; Hayashi, 1991). Disadvantages include fragment size and difficulty in interpretation of gels (Wang *et al*., 2008). Numerous authors found that for fragments bigger than 400 bp, as is the case for *S. cerevisiae*, this technique's discriminatory analysis decreased considerably whereas regions smaller than 350 bp would be sufficient for intraspecies differentiation (Wagner, 2002; Wang *et al*., 2008).

2.4.7.13 Nucleic acid sequence-PCR (NASBA)

This technique is based on the amplification of RNA and not DNA. It was initially described by T. Compton in 1991 and generally involves the usage of three enzymes, AMV reverse transcriptase, RNase H and T7 RNA polymerase (Compton, 1991). Generally the oligonucleotide primers target RNA and deoxynucleotide triphosphate and ribonucleotide triphosphates are included in the amplification reaction mix. Nucleic acid sequence-PCR has not become routine in laboratories but shows potential for the detection of food and beverage yeasts. The technique is fast and takes less time than normal PCR and the development of molecular beacons has allowed for quantification of NASBA products through real-time PCR (Fernandez-Espinar *et al*., 2006)

2.4.7.14 Peptide nucleic acid (PNA) - technology

Peptide nucleic acid (PNA) refers to the use of peptide nucleic acid probes. This technique uses PCR or real-time PCR and the probes are described as DNA mimics (Nielsen *et al*., 1994).The use of this technology has been functional since the early 1990s (Egholm *et al*., 1993; Nielsen *et al*., 1994). PNA can be useful for the detection of point mutations, chromosome analysis and the targeting of species-specific rRNA sequences (Igloi, 1999; Taneja *et al*., 2001). This technique has been applied in the detection of spoilage yeast (*Candida* and *Dekkera*) of the wine industry (Stender *et al*., 2001; Kurtzman, 2006). Advantages include the dilution of samples and the direct probe method. However, a disadvantage is that probes will have to be designed for all yeast species (Kurtzman, 2006).

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2.4.8 DNA microchips

DNA microchips became operational in the early 1990s and have been used ever since and later confirmed as a research tool, especially for understanding wine yeasts. These chips are produced by specialised companies and contain relevant genomic information (DNA, RNA), which are systematic arranged on solid supports, whether it be glass or plastic (Fernandez-Espinar *et al*., 2006). The sample DNA that comes in contact with the chips are usually marked with fluorescent dyes or markers, but can also be labelled by radio-activity or chemio-luminescence. Once in contact with the chips, only chains complementary to those on the chips bind and form patterns of light. These patterns can be read by scanners and the data interpreted. The chips have high specificity and sensitivity, but are relatively expensive (Fernandez-Espinar *et al*., 2006). DNA microchips are widely used for investigating biological processes in organisms. Several studies have used this technology to look at expression levels in genes as well as genomic studies of laboratory and wine yeasts (Cavalieri *et al*., 2000; Hauser *et al*., 2001; Rossignol *et al*., 2003; Rossouw & Bauer, 2009). The use of microchips has led to a better understanding of wine yeasts, commercial or natural isolates, and how they differ from laboratory strains based on specific metabolic and physiological features (Fernandez-Espinar *et al*., 2006). This technique can be used to differentiate between wine yeasts, as not all strains behave the same under wine-making conditions.

2.4.9 Microarray karyotyping

Microarray karyotyping or array CGH (aCGH) has been widely used for genomic research including gene expression patterns, genomic DNA copy number changes and genome rearrangement (Eisen *et al*., 1999; Holloway *et al*., 2002). During microarray karyotyping two separate DNA samples are labelled with two different fluorescent dyes (red-Cy5 & green-Cy3). The two samples are then mixed, hybridised and spotted onto a microarray, whereby each spot is composed of PCR-amplified DNA (Dunn *et al.* 2005). This technique has been used in exploring the copy number changes of genes for species of which the whole genome has been sequenced in relation to other strains of the same species. This provides information on whole or partial chromosome aneuploidy, and non-reciprocal translocations. This technique has been used in *S. cerevisiae* studies to observe the tolerance to low glucose concentrations (Dunham *et al*., 2002). Microarray standard PCR and real-time PCR has been used to verify deletions or amplifications predicted by microarray karyotyping analysis (Lashkari *et al*., 1997a; Perez-Ortin *et al*., 2002; Bond *et al*., 2004). DNA sequencing done by Winzeler *et al*. (2003) validates results obtained by aCGH for rearrangement of single nucleotide polymorphisms. This technique has also been used in studying expression patterns of wine yeast (Backhus *et al*., 2001; Erasmus *et al*., 2003; Rossignol *et al*., 2003). In a further application microarray karyotyping has been useful in researching genomic diversity within the *sensu stricto* group of

S. cerevisiae and the closely related *S. pastorianus* (Lashkari *et al*., 1997b; Perez-Ortin *et al*., 2002; Infante *et al*., 2003).

2.4.10. DNA Sequencing

Major advances in DNA sequencing have provided the platform for understanding primary structures of genes and deduced functions for these genes from previously sequenced information. Various sequencing methodologies have been developed over the past few years, and high-throughput sequencing is bound to become the major molecular tool for strain typing in the near future. Up to now, this technique has been mainly used for the sequencing of specific genome areas after PCR (Fernandez-Espinar *et al*., 2006). Regions of interest for sequencing include the domain D1 and D2 of the 26S gene (Kurtzman & Robnett, 1998), 18S (James *et al*., 1997) and the 5.8S rRNA gene (Las Heras-Vazques *et al*., 2003). Various publications reported the usefulness of amplifying and sequencing ITS regions for rapid identification of fungi including non-*Saccharomyces* (*Candida, Pichia, Hanseniaspora, Torulaspora, Metschnikowia, Saccharomycopsis*) and *S. cerevisiae* yeast (Turenne *et al*., 1999; Arias *et al*., 2002; Mesoud *et al*., 2004; Jespersen *et al*., 2005) from food and beverages. The availability of these amplified and sequenced regions online (http://www.ebi.ac.uk/Blas2/index.html) has also made it easier to assign unknown yeast strains to specific genus or species (Fernandez-Espinar *et al*., 2006). Sequencing has been useful in Sequence characterized amplified region PCR (SCAR-PAR). Whereby strain or species specific bands are cut from gels, reamplified and cloned. These inserts are sequenced and subsequently primers can be designed accordingly for further investigation (Fröhlich & Pfannebercker, 2007).

Genome sequencing has evolved over last few years and current methods include the whole-genome shotgun approach and full genome sequencing. During the shotgun approach, genomic DNA is isolated and fragmented into smaller pieces and sequenced by using a chain termination method. As multiple overlapping reads from fragments are created, computer software programmes use the overlapping regions to create a continuous sequence. This method was the precursor that led to full genome sequencing. Currently full genome sequencing is done by techniques such as pyrosequencing, SMRT sequencing and nanopore technology. A whole host of commercial companies currently compete with various platforms and these include Illumina, Knome, Sequenom and Pacific Biosciences to name a few.

2.5 Conclusion

The techniques discussed above are focused on identifying or characterizing yeasts or other microorganisms from various research fields. In the past phenotypical and morphological test methods were used, which has become out-dated and usually considered labour intensive and expensive. In comparison a more molecular approach represents an enhancement in terms of

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resolution and processing time. The key aspects of these molecular techniques when identifying organisms from various genera or specific species level include speed and ease of use, while still obtaining reproducible and accurate results. The use of one or more technique has become a general or standard practice, as these techniques usually complement or supplement results. Each technique is based on a specific method of analysis from DNA fingerprinting, mitochondrial DNA, chromosomal DNA patterns, restriction analysis of various parts of the genome to newer technologies such as microarrays and peptide nucleic acid probes which divert from the normal DNA based methods. Laboratories and research institutes generally consider advantages and disadvantages before selecting one of these techniques. Physiological or morphological methods of old are still useful but advances in technology have given more scope to understanding the whole genome of an organism, whether it is bacteria or yeasts. Future trends will include the use of –omic (transcriptome, metabolome & proteome) tools for the identification of specific species or strains. Omic system-wide approaches have already been helpful in studying functional complex cellular networks within laboratory yeast strains and industrial yeast

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Chapter 3

Research results

Molecular typing of wine yeasts: Evaluation of typing techniques and establishment of a database

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RESEARCH RESULTS

3.1 INTRODUCTION

Saccharomyces sensu stricto generally refers to yeasts strains associated with the food and beverage industries, and several strains serve as important scientific model systems (Rainieri *et al*., 2003). This taxonomic complex includes several biological species such as: *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* and the recently described *S. arboricolus* (Vaughan-Martini & Martini, 1987; Naumov *et al*., 2000; Wang *&* Bai, 2008). Frequently, *S. cerevisiae*, *S. bayanus* and *S. pastorianus* are associated with anthropic environments because of strong fermenting capabilities (Naumov *et al*., 2000), and *S. cerevisiae* strains are mainly responsible for alcoholic fermentation in the wine industry (Pretorius, 2000). However, the origin of *S. cerevisiae* has been a topic of hot debate. This species has been presumed to be absent from grapes and vineyard soils (Martini & Vaughan-Martini, 1990), while it has been proposed that they occur naturallly on plant fruits (Sniegowski *et al*., 2002). Domestication of *S. cerevisiae* species in anthropic environments like cellars has been proposed, which in turn would lead to transference to vineyards (Naumov, 1996). The evolution of wine yeast strains has been studied by various researchers investigating topics such as genomic characteristics, stress adaptation, gene expression variability and interspecific hybridisation between species within the *Saccharomyces sensu stricto* group. Interspecific hybridisation in particular has been proposed as a feature of evolution of strains within this group (Querol *et al.*, 2003; Sicard & Legras, 2011).

Traditionally, characterisation and identification of *Saccharomyces* and other yeast species relied on morphological and physiological tests, that are time-consuming and laborious and sometimes of doubtful taxonomic usefulness (Deak, 1995). It has also become apparent that many biotechnologically relevant strains within the *Saccharomyces sensu stricto* complex are phylogenetically similar and cannot be differentiated with most conventional methods (Rainieri *et al*., 2003). Molecular methods have provided a new approach for identification and characterisation of these yeasts. These molecular techniques include, DNA base composition (GC content) (Yarrow & Nakase, 1975), DNA reassociation (Vaughan-Martini & Kurtzman, 1985), and CHEF karyotyping (Oliveira *et al*., 2008). Polymerase chain reaction (PCR)-technology has been widely used with techniques such as interdelta region amplification (Legras & Karst, 2003), mitochondrial DNA (mtDNA) restriction analysis (Fernandez-Espinar *et al*., 2001), rDNA spacer regions PCR-RFLP analysis (Fernandez-Espinar *et al*., 2000), randomly amplified polymorphic DNA (RAPD) (Fernandez-Espinar *et al*., 2003), amplified fragment length polymorphism (AFLP) (Esteve-Zarzoso *et al*., 2010), simple sequence repeats (SSR) (Hennequin *et al*., 2001) or microsatellite primers (Baleiras-Couto *et al*., 1996), denaturing

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gradient gel electrophoresis (DGGE) (Cocolin *et al*., 2000), gene sequencing of rDNA spacer regions (ITS and D1/D2) (Kurtzman & Robnett, 1998), multigene sequence analysis (MLSA) (Kurtzman & Robnett, 2003) and the use of species-specific primers (Josepa *et al*., 2000, Huang *et al*., 2008). The disadvantages of these techniques are that they may be complicated and time-consuming and significant optimisation maybe required.

Although all these methods are available there are still disputes regarding the identity of strains produced for the wine industry. Anecdotal evidence has been reported that strains may have been duplicated or illegally produced to gain market share. After thorough investigation, no commercial database could be found to easily compare molecular profiles of wine yeast strains.

This study investigated the use of CHEF, PCR of interdelta regions and multiplexing of microsatellite primers to differentiate between 62 wine and two reference yeast strains. *MET2* gene analysis and species-specific PCR, to evaluate oenological designation of these strains, were also investigated. This study led to the establishment and evaluation of a database, containing specific libraries for CHEF and interdelta regions.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains

Sixty-two commercial wine yeast strains from various manufacturers/suppliers and two reference strains were used in this study (Table 3.1). Wine yeast strains included *S. cerevisiae*, *S. bayanus* and hybrid strains as labelled by the producer.

3.2.2 Isolation and cultivation of yeast strains

In general cultivation, one gram of each actively dried wine yeasts (ADWY) was suspended in 9 mL of saline solution (8.5 g sodium chloride per litre of distilled water) from which an dilution series was made and plated out on yeast peptone dextrose (YPD) agar and lysine media (BioLab, Merck, SA). Lysine media was used to screen for possible non-*Saccharomyces* yeasts contaminants. These plates were incubated at 30°C for 3-4 days. Colonies from the highest dilution were counted and the numbers recorded. Ten colonies were randomly selected and stored in 80% glycerol at -80°C until required.

For CHEF karyotyping, 100 mL YPD broth were inoculated with single colonies (wet cultures) or one gram of the active dried yeast (ADY) and incubated at 28°C for 17-19 h (late logarithmic phase) with agitation prior to chromosomal DNA extraction.

For PCR, a single colony of the purified yeast strains (three biological repeats) was inoculated in 5 ML YPD broth and incubated at 30°C for 24 h.

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Table 3.1 Reference and commercial wine yeasts used in this study.

Commercial/ Reference strains	Strain identity[1]	Wine styles	Manufacturer/Reference
Saccharomyces bayanus	*S. bayanus*	-	CBS 380
Saccharomyces cerevisiae	*S. cerevisiae*	-	CBS1171
VIN 7	*S. cerevisiae*	white wines	Anchor Yeast, South Africa
VIN 13	*S. cerevisiae*	white wines	
VIN 2000	*S. cerevisiae*	white wines	
N96	*S. cerevisiae*	white wines	
NT 45	*S. cerevisiae*	red wines	
NT 50	*S. cerevisiae*	red wines	
NT 112	*S. cerevisiae*	red wines	
NT 116	*S. cerevisiae*	white/red wines	
NT 202	*S. cerevisiae*	red wines	
228	*S. cerevisiae*	brandy/semi-sweet	
WE 14	*S. cerevisiae*	semi-sweet	
WE 372	*S. cerevisiae*	red wines	
BM 4X4	*S. cerevisiae ex bayanus*	white/red wines	Lallemand, Canada
BM 45	*S. cerevisiae*	red wines	
Cross Evolution	*s. cerevisiae*	white/rosé wines	
EC 1118	*S. cerevisiae ex bayanus*	white wines	
Enoferm BDX	*S. cerevisiae*	red wines	
Enoferm Syrah	*S. cerevisiae*	red wines	
ICV D-47	*S. cerevisiae*	white wines	
ICV D80	*S. cerevisiae*	red wines	
Enoferm QA23	*S. cerevisiae ex bayanus*	white wines	
PDM	*S. cerevisiae ex bayanus*	white wines	
Uvaferm 43	*S. cerevisiae ex bayanus*	red wines	
Fermicru 4F9	*S. cerevisiae*	white/rosé wines	DSM, France
Fermicru VR5	*S. cerevisiae*	red wines	
Fermicru LV CB	*S. cerevisiae ex bayanus*	white wines	
Fermichamp	*S. cerevisiae ex bayanus*	white wines	
Cabernet Sauvignon	*S. cerevisiae*	red wines	
Chardonnay	*S. cerevisiae*	white wines	
Merlot	*S. cerevisiae*	red wines	

[1] Strain identity as labelled by manufacturer.

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Table 3.1 Reference and commercial wine yeasts used during the study (continued)**.**

Commercial/ Reference strains	Strain identity[1]	Wine styles	Manufacturer/Reference
Actiflore PM	*S. cerevisiae var. bayanus*	white wines	Laffort, France
Actiflore BO 213	*S. cerevisiae var. bayanus*	white wines	
Zymaflore F15	*S. cerevisiae*	red wines	
Zymaflore FX10	*S. cerevisiae*	red wines	
Zymaflore VL3	*S. cerevisiae*	white/rosé wines	
Zymaflore X5	*S. cerevisiae*	white/rosé wines	
Zymaflore X16	*S. cerevisiae*	white/rosé wines	
IOC B 2000	*S. cerevisiae*	white wines	Institute Oenoligique de Champagne (IOC), France
IOC 18-2007	*S. cerevisiae var. bayanus*	white/red wines	
IOC Primrouge R9001	*S. cerevisiae*	red wines	
IOC R-9002	*S. cerevisiae*	red wines	
A3B	*S. bayanus*	white/red wines	AEB (Pascal Biotechnologies)
Arome plus	*S. cerevisiae*	white wines	
Fermol Sauvignon	*S. cerevisiae*	white wines	
Fermol Chardonnay	*S. cerevisiae*	white wines	
Super16	*S. cerevisiae*	white/red wines	
NWS Chardonnay	*S. cerevisiae*	white wines	Intec International Technologies
NWS Merlot	*S. cerevisiae*	red wines	
NWS Pinot	*S. cerevisiae*	red wines	
Enartis *S. bayanus*	*S. bayanus*	white/rosé wines	Enartis, Portugal
Enartis *S. cerevisiae*	*S. cerevisiae*	white/red wines	
AWRI 796	*S. cerevisiae*	white/red wines	Maurivin, Australia
AWRI 1503	*S. cerevisiae/kudriavzevii*	white/red wines	
AWRI Fusion	*S. cerevisiae/cariocanus*	white/red wines	
BP 725	*S. cerevisiae*	red wines	
Elegance	*S. cerevisiae*	white wines	
Maurivin B	*S. cerevisiae*	red wines	
Merit.ferm	*S. cerevisiae*	red wines	CHR Hansen, Denmark
SLC	*S. cerevisiae*		Dal.cin uk ltd.london
Enodoc RJ11	*S. cerevisiae*		
Eno Arome K7	*S. cerevisiae*	white/rosé wines	Springer Oenologie, Germany
CK S102	*S. cerevisiae*	white wines	

[1] Strain identity as labelled by manufacturer.

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3.2.3 DNA Extraction

Chromosomal DNA for CHEF was prepared using an embedded agarose technique by Carle and Olson (1985). Day one, cells were harvested from the YPD broth by centrifugation, subsequently washed twice with 10 mL of 10 mM EDTA (pH 7.5) and centrifuged at 4°C (8000 rpm, 10 min). Harvested cells were than resuspended in 3 mL of 50 mM EDTA (pH 7.5). The suspension was than mixed with 1 mL Solution I (10 mL SCE buffer (1.0 M sorbitol/0.1 M sodium citrate/60 mM EDTA (pH 5.8)/250 mL distilled water), 0.5 mL of 2-mercaptoethanol and 10 mg Lyticase (Sigma-aldrich) and 5 mL of 1% low-gelling temperature agarose (prepared with 0.125 M EDTA, pH 7.5) and decanted into a small Petri dish and allowed solidify. Once solidified, the agarose was cut into small plugs and stored with 5 mL of Solution II (0.45 M EDTA/pH 9.0, 10 mM Tris-HCl/pH 8.0, 7.5% vol/vol 2 mercaptoethanol) in a small glass tube (McCartney) and incubated at 37°C for 24 h. Day two, Solution II was replaced with 5 mL Solution III (0.45 M EDTA/pH 9.0, 10 mM Tris-HCl/pH 8.0/1% sodium N-lauroylsarcosinate/1 mg/mL proteinase K (Sigma-Aldrich)) and incubated at 50°C for 48 h (Carle & Olson, 1985; van der Westhuizen *et al*., 1999). After 48 h solution III was decanted, replace by 5 mL of 0.5 M EDTA (pH 9.0) and stored at 4°C for further use.

Two millilitres of the overnight yeast culture was used for DNA extraction for PCR. Genomic DNA was extracted according to the method of Hoffman and Winston (1987).

3.2.4 Amplification conditions

Interdelta region amplifications were carried out in 50 µl reaction volumes containing 20 ng yeast DNA, 10x reaction buffer (Southern Cross Biotechnologies PTY (LTD), 25 mM $MgCl_2$, 10 µM of each oligonucleotide primer, 2.5 mM of each dNTP and 0.5 U Super-Therm Taq polymerase (Southern Cross Biotechnologies PTY (LTD). Primers used are listed in Table 3.2. All PCR reactions were performed with a BioRad icycler. Amplifications were performed using the following programme: 4 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 48°C and 90 s at 72°C and a final elongation step of 10 min at 72°C.

Microsatellite amplifications were done in 50 µl reaction volumes containing 10-20 ng of yeast genomic DNA, 5x reaction buffer (Promega, Madison WI, USA) 25 mM $MgCl_2$, 10 µM of each oligonucleotide primer (Table 3.2), 2.5 mM of each dNTP and 0.5 U GoTaq polymerase (Promega, Madison WI, USA). Primers used are indicated in Table 3.2. Amplification reactions were performed using the following programme: 4 min at 95°C followed by 30 cycles of 15 s at 95°C, 45 s at 55°C and 90 s at 72°C and a final elongation step of 4 min at 72°C.

MET2 gene amplifications were performed in a BioRad icycler using synthetic oligonucleotide primers for *MET2* (Table 3.2) as described by Hansen & Kielland-Brandt (1994). Amplifications were carried out in 50 µl reaction volumes containing 10 ng DNA, 5x Gotaq reaction buffer (Promega, Madison WI, USA), 25 mM $MgCl_2$, 10 µM of each primer, 2.5 mM of each dNTP and 0.5 U Gotaq polymerase (Promega, Madison WI, USA). The cyclic program

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included the following: 1 min at 94°C followed by 25 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C and a final elongation step of 10 min at 72°C. PCR products were then subsequently digested with *EcoRI* or *PstI*.

Species-specific primers amplification were performed in 50 µl reaction volumes containing 1-10 ng genomic DNA, 10x reaction buffer, 1.5 mM $MgCl_2$, 1 µM of each primer (Table 3.2), 2.5 mM of each dNTP and 0.5 U GoTaq polymerase.

For the SB and SC primers the following thermocyclic programme was used: 5 min at 94°C followed by 30 cycles 30 s at 94°C, 1 min at 50°C and 1 min at 72°C and a final elongation step of 7 min at 72°C.

For the YB1f/YB2r primers the following cyclic programme was used: 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and a final elongation step of 10 min at 72°C.

For the SpeOPT18SBAY-F2/R2 primers the following programme was used: 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 66°C and 2 min at 72°C and a final elongation step of 7 min at 72°C.

3.2.5 Electrophoretic separation

A CHEF DRII electrophoretic system was used for separation of chromosomal DNA. These separations were carried out in 20 cm, 1.2 % agarose (Seakem® GTG®, Lonza, Rocklands, USA) gels made in 0.5 x TBE buffer (pH 8.4) at a constant temperature of 14°C. Electrophoretic gels were run for 26 hours at a constant voltage of 200 V. Pulse duration for the first 15 hours was 60 s and for the following 11 hours it was 90 s. VIN 13 was used as a standard reference strain and loaded on the outer lanes of each gel. Gels were stained in 500 mL, 0.5 x TBE buffer with 15 µl ethidium bromide (10 mg/mL) for 0.5 hours and destained in 500 mL of the same buffer for 0.75 hours.

Interdelta region and microsatellite products were separated on 2% gels submitted to 90 V for 2.5 h in 0.5x TBE buffer.

*MET*2 gene and resulting restriction fragments were analysed on 1.8% agarose gels and submitted to 120 V for 1.5 h in 0.5x TBE buffer.

Species-specific amplifications were separated on 1.5 % agarose gels and subjected to 85 V for 2.5 h in 0.5x TBE buffer.

All agarose gels used for visualisation of PCR products contained 15 µl of a 5000x GelRed™ (Biotium). Generuler 100 bp Plus DNA (Fermentas) was used as a standard marker/ladder on PCR gels. Additionally a λDNA *EcoRI/HindIII* marker was used for the SPEOPT18SBAY f2/r2 gels.

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TABLE 3.2. Primers used in this study.

Primers	Sequences	References
Delta elements		
δ1	CAA AAT CAC CTA TAT CT	Ness *et al*. (1993)
δ2	GTG GAT TTT TAT TCC AAC	
δ12	TCA ACA ATG GAA TCC CAA C	Legras & Karst (2003)
δ21	CAT CTT AAC ACC GTA TAT GA	
Microsatellites		
GTG5	GTG GTG GTG GTG GTG	Britos dos Santos *et al*., 2007
M13-BACTERIOPHAGE	GAG GGT GGC GGT TCT	
Species-specific		
MET2 FORWARD	CGG CTC TAG ACG AAA ACG CTC CAA GAG CTG G	Hansen & Kielland-Brandt (1994)
MET2 REVERSE	CGG CTC TAG AGA CCA CGA TAT GCA CCA GGC AG	
SC1	AAC GGT GAG AGA TTT CTG TGC	Josepa *et al.* (2000)
SC2	AGC TTG CAG TAT TCC CAC AG	
SB1	GCT ATT CCA AAC AGT GAG ACT	
SB2	CAG TTG GCA GTA TTC CCA CTA	
YB1F	AAC GAT ATT AGA ACA TTC CTC CAC	Torriani *et al*. (2004)
YB2R	GCT GTT GCA GAC ATA GTG TG	
SPEOPT18SBAY-F2	TTG AAG GAA CAA CAG TGG AAG	Huang *et al*. (2008)
SPEOPT18SBAY-R2	CCC GAA AAA CAG GAG TGA C	

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3.2.6 Numerical analysis of CHEF karyotyping and interdelta PCR

Gels were subsequently visualised and scanned using a Gel Doc XR apparatus (BioRad Laboratories, Richmond, USA) and the profiles were saved. Normalisation of gels and comparison of profiles were done using FPQuest™ software (BioRad Laboratories, Richmond, USA). The normalised electrophoretic patterns were grouped, and similarity (s) and cophenetic correlations (cc) were obtained, using the Dice coefficient. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cophenetic correlation is a measure of how faithfully a dendogram preserves the pairwise distance between the original unmodeled data points.

3.2.7 Database creation and analysis

FPQuest™ software (version 4.5) was used to normalise gel profiles and create a database containing three fingerprint types, CHEF karyotypes and profiles of two delta primer sets. Gel electrophoresis system CHEF DRII (BioRad Laboratories, Richmond, USA) was used during optimisation and evaluation. Commercial yeast strains used for the evaluation of the database are given in Table 3.3.

TABLE 3.3 Yeast strains used for testing the accuracy of the database and corresponding test organism (TO) codes.

Yeasts	Test organism
EC 1118	Unknown 1
NT 45	Unknown 2

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3.3 RESULTS AND DISCUSSION

3.3.1 Analysis of chromosomal banding patterns by CHEF karyotyping

In this study, 62 commercial wine yeasts were randomly selected from various manufacturers and two reference strains, *S. cerevisiae* (CBS 1171) and the cryophilic *S. bayanus* (CBS 380), were used (Table 3.1). Karyotypes obtained for the reference and selected wine yeast strains are shown in Fig. 3.1. As a quality control and an optimisation tool, ADY form of strains were compared to colonies (wet cultures) isolated from the commercial packets. Colonies yielded better CHEF results compared to the ADY forms (Fig. 3.1d), because the growth from colonies could be controlled and monitored when entering early logarithmic phase. Karyotypes generated from the ADY forms were generally over exposed because of the high DNA concentrations as well protein contamination. This made it difficult to distinguish chromosomal polymorphisms, especially when chromosomes were close together. However, this problem could be rectified by rehydrating ADY forms and inoculating a diluted suspension into fresh growth media.

Sixty four different CHEF karyotypes were obtained, indicating a very high resolution of the methodology. The data also suggest that some commercial strains share chromosomal DNA from both reference strains indicating possible genetic relatedness towards both species. Phylogenetic relatedness for the 62 commercial strains and reference strains based on chromosomal arrangement were also indicated. A number of yeast strains, EC 1118, QA23, PDM, Actiflore PM, Fermicru 4F9, NWS Pinot, NWS Chardonnay and N96, generated similar karyotypes and only differed by a single chromosomal polymorphism. Three distinct groups (Groups I, II and III) were delineated at 48.5%. Group I contained eleven yeasts, which included the *S. bayanus* reference strain. The *S. bayanus* reference strain had a 99% cophenetic correlation to three yeasts, *i.e*. IOC B2000, CK S102 and Eno Arome K7. From group I, it is noticeable that there is no general grouping or clustering of the commercial strains with the *S. cerevisiae* and *S. bayanus* reference strains. Cardanali & Martini (1994) reported that strains from the same species usually grouped together. However, their study did not include commercial wine yeast strains.

Within group I, it was noticeable that cophenetic correlations ranged from 76-100% whereas similarity ranged from 49.9-88%. However, it is important to look at the similarity values and cophenetic correlation coincidently to determine if yeasts are indeed similar. Based on these factors two commercial yeasts, BM 45 and BM 4X4 (Lallemand) had similar banding patterns. The dendogram show a similarity value of 78%, with a single chromosomal polymorphism difference. According to the producer, BM 4X4 is a mixed culture of BM 45 and another yeast. However, isolation and analyses of several colonies yielded only one profile, with the only possible explanations being that these strains have similar karyotypes or that one strain dominated the mixture.

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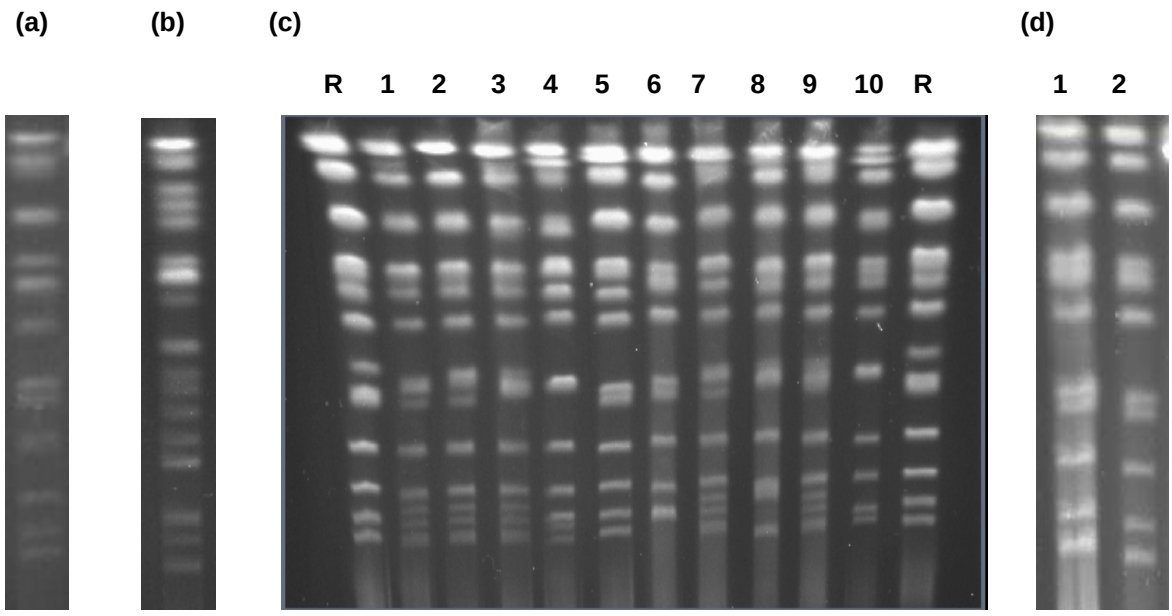

FIGURE 3.1

Contour-clamped homogeneous electric field gel electrophoreses (CHEF) karyotypes of reference and selective commercial wine yeast strains. (a) Karyotype of *S. cerevisiae* CBS 1171, reference strain. (b) Karyotype of *S. bayanus* CBS 380, reference strain. (c) Gel reference strain (R): VIN 13, lane 1: N96, lane 2: EC 1118, lane 3: QA23, lane 4: WE 14, lane 5: 228, lane 6: Merit.ferm, lane 7: NWS Pinot, lane 8: NWS Merlot, lane 9: NWS Chardonnay, lane 10: Cabernet Sauvignon, Reference strain (R): VIN 13.
(d) Comparison of the active dried yeast (ADY) and colony (wet culture), lane 1: ADY, lane 2: colony.

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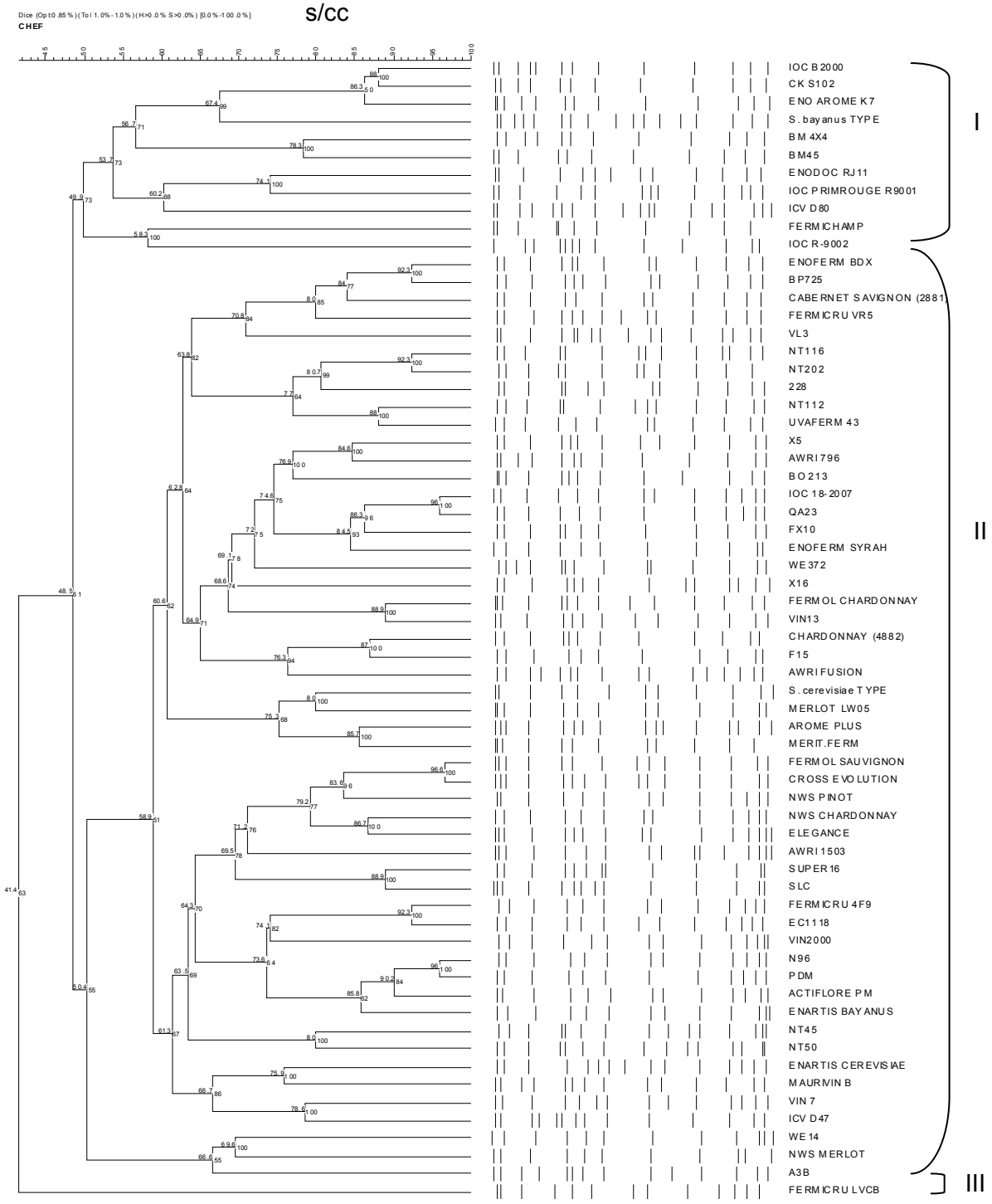

FIGURE 3.2

Dendogram showing the clustering of 64 yeast strains obtained by numerical analysis of CHEF karyotypes. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarity (s) and cophenetic correlations (cc) between strains were calculated based on the Dice coefficient.

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Two other strains within Group I, Enodoc RJ11 and Primrouge R9001, had a 100% cophenetic correlation with similarity at 74%.

Group II contained 52 yeasts had a 50.4 % similarity and 61 cophenetic correlation to Group I. In Group II further delineation could be seen at 50% similarity and 55% cophenetic correlation where three subclusters are visible. Generally this group had high similarity values and cophenetic correlations. Group II also included the *S. cerevisiae* reference strain that clustered with Merlot LW05. Analysis of group II yielded six pairs of commercial yeast strains with similar karyotypes, *i.e*. N96 and PDM; Enoferm BDX and BP 725; Fermol Chardonnay and VIN 13; NT 116 and NT 202; IOC18-2007 and Enoferm QA23; and Fermol Sauvignon and Cross Evolution. Similarity ranged from 92.3 to 96%. Figure 3.2 show the grouping of several commercial yeast strains from the same manufacturer. This is evident in the grouping of strains from Anchor Yeast, where NT 45 and NT 50 grouped together, and NT 112, NT 116, 228 and NT 202 formed a separate subgroup. Similarity within this grouped varied from 50 to 100 %. Fermicru LVCB clustered on its own (group III) at 41.4% similarity and 63% cophenetic correlation to groups I and II. There is also no correlation between these commercial strains based on the wine style (Table 3.1). Results showed that CHEF karyotyping can be used to differentiate between commercial wine yeasts at strain level.

3.3.2 Evaluation of interdelta regions

Two interdelta primer sets, delta1-2 and delta12-21, were used to distinguish between the 64 yeast strains. The amplified profiles were used to compare the reference strains of *S. bayanus* and *S. cerevisiae* to the commercial wine strains. Various PCR conditions were optimised and included assessing the variation in DNA concentrations, primer concentrations and annealing temperatures (42 to 48°C). Optimal results were achieved at a temperature of 48°C and a DNA concentration of 20 ng/µl. At this temperature reliable and comparable results were obtained. Amplifications were also done in biological triplicate to evaluate the consistency of the method. The use of colonies or genomic DNA was also evaluated. Both DNA sources yielded satisfactory quality; indicating that colony PCR is an appropriate methodology. Figure 3.3 shows the electrophoretic profiles obtained with delta1-2 and delta12-21, respectively. The delta1-2 primer set yielded fewer bands than the delta12-21 primer set. Band sizes ranged from 100 to 3000 bp. During the amplification of the 64 profiles based on delta1-delta2, there were 1 to 3 band patterns consistently in the 300 to 500 bp region (demarcated in Fig. 3.3).

Amplifications using delta 1-2 yielded 59 distinct profiles for the 64 strains tested (Fig. 3.4). Five groups could be delineated at 33% similarity with ICV D-47 (Group IV) and NWS Pinot (Group V) clustering separately from the other strains (Fig. 3.4). ICV D-47 yielded two bands, and NWS Pinot, one band during amplification. The majority of the strains were clustered in Group I, which include both reference strains. There was no resemblance of the delta1-2 groups to that of CHEF karyotyping groups. Delta1-2 results showed high phylogenetic relationships for

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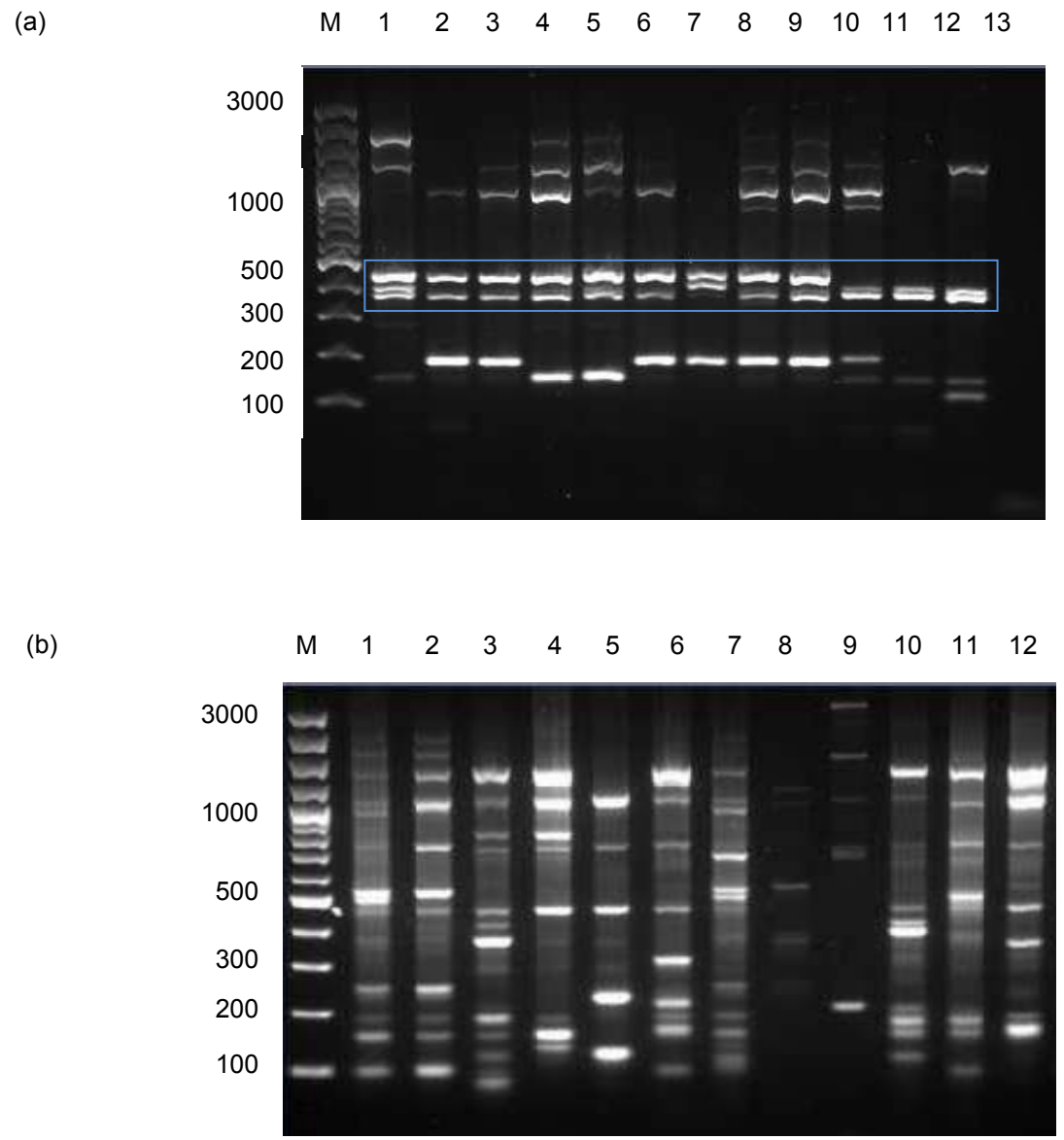

FIGURE 3.3

Interdelta amplification products in commercial wine yeast strains. (a) Amplification with delta1-2 (Ness *et al*., 1993), Marker- Generuler 100 bp Plus DNA marker, lane1: VIN 7, lane 2: VIN 13, lane 3: VIN 2000, lane 4: NT 45, lane 5: NT 50, lane 6: NT 112, lane 7: NT 116, lane 8: NT 202, lane 9: N96, lane 10: 228, lane 11: WE 14, lane 12: WE 372, lane 13: NWS Pinot. (b) Amplification with delta12-21 primers (Legras & Karst, 2003), Marker- Generuler 100 bpPlus DNA marker, lane 1: Fermol Sauvignon, lane 2: Fermol Chardonnay, lane 3: A3B, lane 4: Arome Plus, lane 5: SUPER16, lane 6: Fermicru VR5, lane 7: Fermicru 4F9, lane 8: Fermichamp, lane 9: LV CB, lane 10: Merlot, lane 11: Cabernet Sauvignon, lane 12: Chardonnay.

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the commercial strains with a number of 100% correlations, similarity and cophenetic, being indicated, *i.e*. AWRI 796 and BP 725, N96 and PDM, NT 112 and VIN 2000, CK S102 and Eno Arome K7, and AWRI 1503 and Elegance. Interestingly, CK S102 and Eno Arome K7 also yielded similar CHEF karyotypes, suggesting genetic relatedness of these two strains. The genetic relatedness of BM 45 and BM 4x4 (Group II), as with CHEF karyotyping was also confirmed eventhough the delta1-2 primers revealed clear differences between these strains.

Importantly, there was no specific correlation of these groups with the groups obtained through CHEF karyotyping, demonstrating the limits of these technologies to clearly assess strain relatedness. The reference strains grouped together in group I at similarity of 41%. The profiles obtained from delta1-2 primer set suggest that yeasts from the some manufacturers are closely related. This evident in the case of AWRI 796 and BP 725 (Maurivin), as well as clustering of Anchor strains NT 45, NT 50 and NT 202, while VIN 7, VIN 2000, VIN 13 and NT 112 (Anchor Yeast) all clustered together. The *S. bayanus* reference (CBS 380) generated a distinct profile in comparison with the commercial stains. The delta1-2 results confirmed the results obtained with CHEF karyotyping. Strains that had similar to identical CHEF karyotypes had similar delta profiles. However, overall clustering of strains was different for the two techniques. Amplifications using delta primers only focuses on a specific area on a chromosome(s) and this could be the reason for different clustering.

Figure 3.5 illustrates the cluster analysis for delta12-21 primer set. Amplifications using delta12-21 yielded 62 distinct profiles. Cluster analysis of Fig. 3.5 showed a delinearisation of three groups (I, II, III). Lower similarity values were evident throughout the dendogram but many more delineations were visible for this primer set. With regard to separation according to species, the *S bayanus* reference strain (CBS 380) and Fermicru LVCB formed a cluster (Group III) on their own with a 37.5 % similarity value with cophenetic correlation at 100% between the two strains. The rest of the 96 % of strains (Group I) were delineated with the *S. cerevisiae* reference strain (Group II). However, group III showed some resemblance to that of group III of the electrophoretic karyotyping with commercial strain Fermicru LVCB (DSM) found in both groups.

As with delta1-2, amplification with delta12-21 illustrated 100% similarity and cophenetic correlations between some strains. These include: CK S102 and Eno Arome K7. Commercial strains such as IOC 18-2007, NWS Pinot and NWS Chardonnay yielded similar to identical profiles. Anchor commercial strains, NT 112, NT 202, NT 116, NT 45, VIN 2000, and NT 50, again showed similar results as with delta1-2. Cluster analysis indicated that N96, QA23, and EC 1118 had a similarity of 83.8%. Bradbury *et al*. (2005) reported that QA23 and EC 1118 to be similar in their study of trinucleotide microsatellites. The strains, Fermol Sauvignon, Fermol Chardonnay, Fermicru 4F9, Enartis *S. bayanus*, Actiflore PM had 81% similarity with similar results being obtained for the Fermol yeasts during delta1-2 amplifications. BM 45 and BM 4X4

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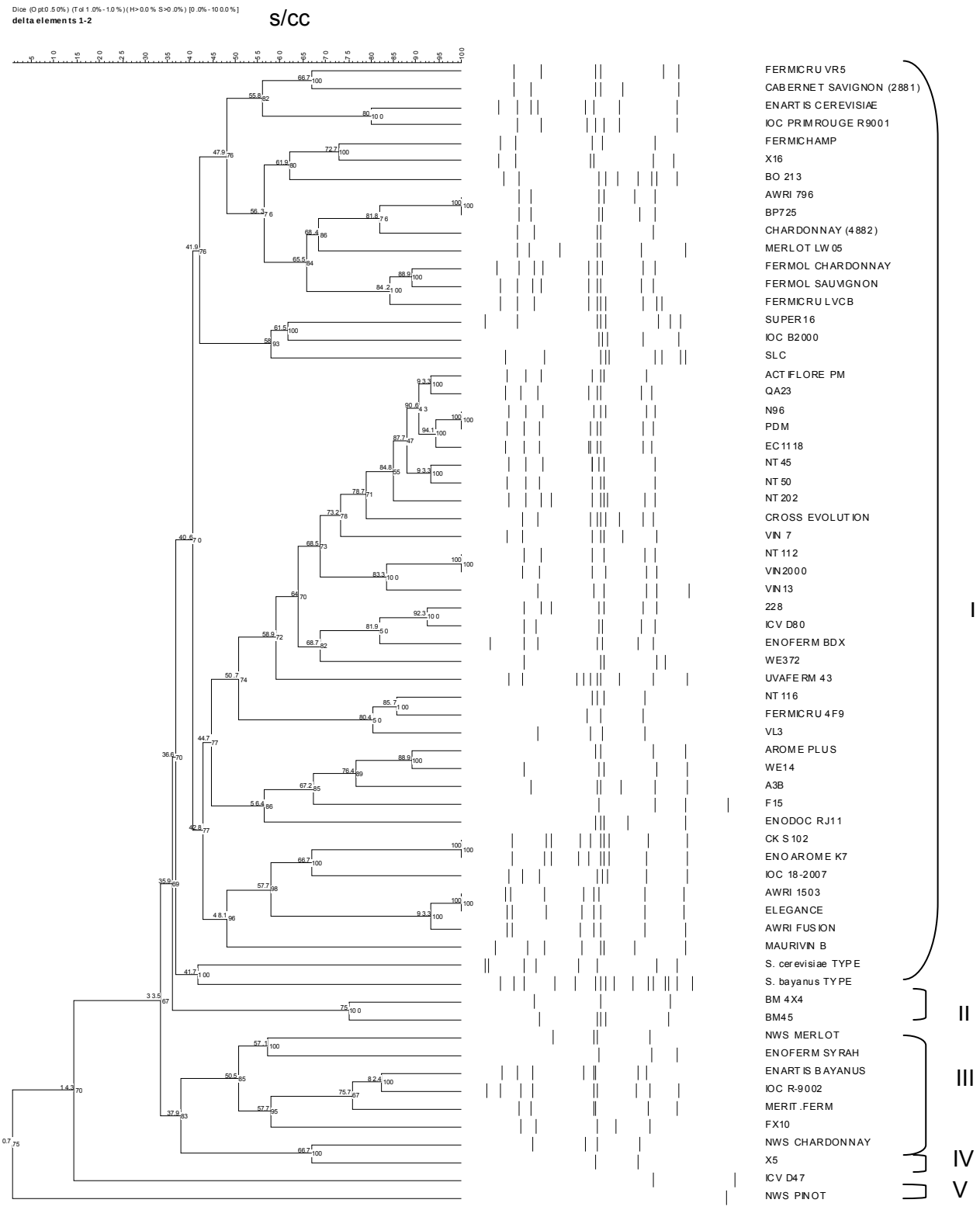

FIGURE 3.4

Dendogram showing the clustering of 64 yeast strains obtained by numerical analysis of delta1-2 profiles. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarity (s) and cophenetic correlations (cc) between strains were calculated based on the Dice coefficient

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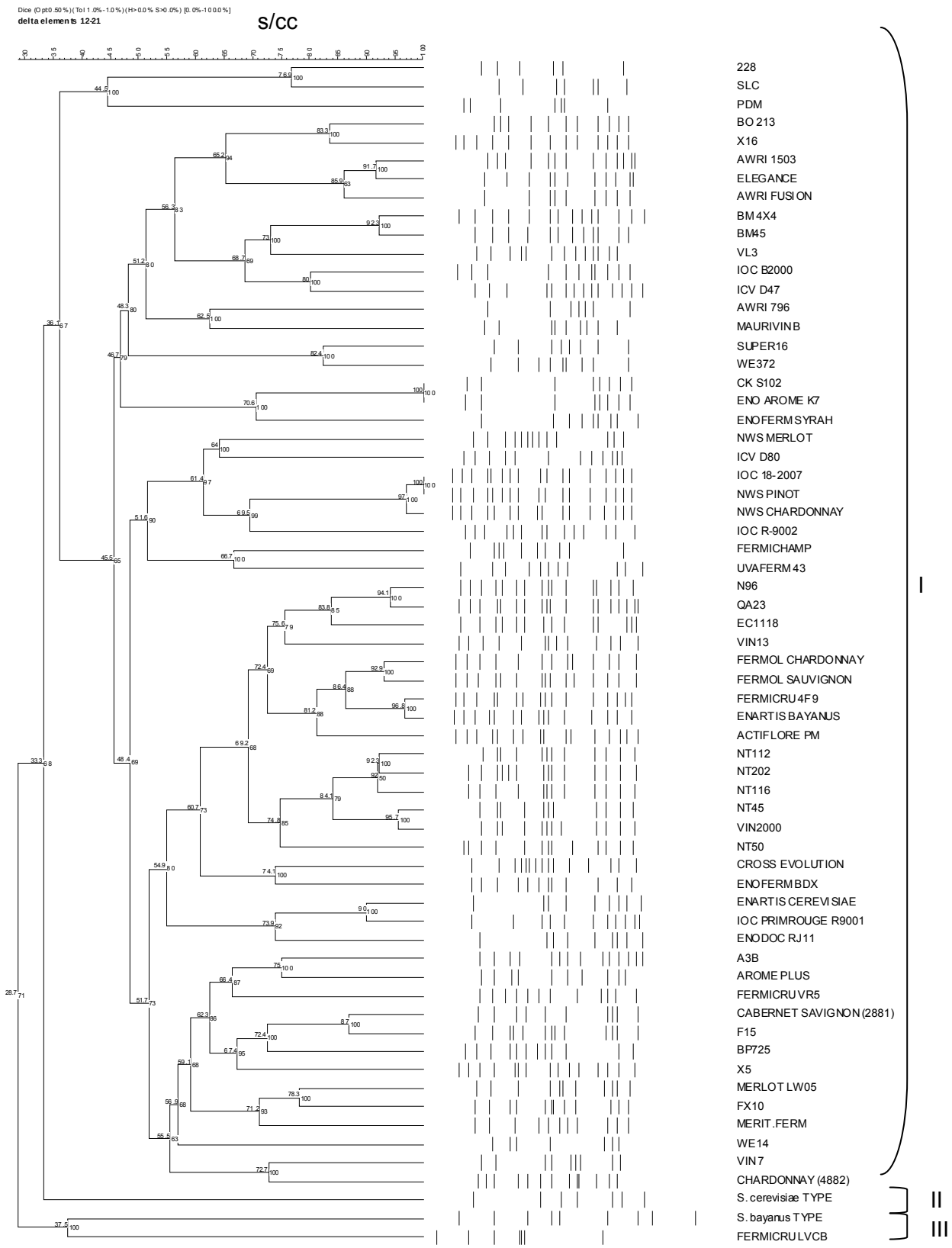

Figure 3.5

Dendogram showing the clustering of 64 yeast strains obtained by numerical analysis of delta12-21 profiles. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarity (s) and cophenetic correlations (cc) between strains were calculated based on the Dice coefficient

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were shown to be genetic closely related by electrophoretic karyotyping, delta1-2 primers and delta12-21. Enodoc RJ11, Enartis *S. cerevisiae* and IOC Primrouge R9001 also had very similar profiles (Fig. 3.5).

3.3.3 Evaluation of multiplex-microsatellite PCR

The RAPD-PCR profiles using GTG and M13 in a multiplex-PCR of selected yeasts are shown in Fig. 3.6a and b. During this investigation the multiplex-PCR of these two primers could only detect differentiation between *S. bayanus* and S*. cerevisiae* species. Eight commercial strains, PDM (Lallemand), Actiflore PM and Fermichamp, characterised as *S. cerevisiae (ex bayanus)* or as *S. bayanus* by manufacturers, elucidated the absence of this band.The size of bands ranged from 100 to 3000 bp and yielded between 6 and 18 bands (min. and max. number of bands). Some “ghost bands” were generated in the 100 to 500 bp range. Generally, all profiles resembled that of the *S. cerevisiae* reference strain (Fig. 3.6b, lane 1), except the *S. bayanus* reference strain, which yielded a unique nine band profile (Fig. 3.6a, lane 14). Two commercial wine strains yielded a 900 bp band, which is also found in the *S. bayanus* reference strain. VL3 and Fermicru 4F9 (lanes 3 and 8, respectively in Fig. 3.6a) distinctly differed from the rest of the commercial wine yeasts. These two profiles share about 88% similarity or homology. In 55 of the 64 yeast studied, a ±730 bp band was observed, which was not present in the *S. bayanus* reference strain, but is discernible in the *S. cerevisiae* reference strain. However, eight commercial wine yeast strains did not have this ±730 bp band and included yeasts from five different manufacturers namely; Fermol Sauvignon, Fermichamp, NWS Pinot, NWS Chardonnay, Actiflore PM, Zymaflore X5, PDM and Uvaferm 43.

The packaging labels identified four of these strains (Actiflore PM, Fermichamp, PDM and Uvaferm 43) as *S. bayanus or S. cerevisiae* (*ex. bayanus*). This suggests that this band is specific to *S. cerevisiae* strains and could be utilised to differentiate *S. cerevisiae* from *S. bayanus* strains. Various authors reported on the microsatellite, GTG_5 (Baleiras Couto *et al.* 1996; Naumov *et al.* 2000; Xufre *et al.* 2000) and the bacteriophage M13 primer (Andrighetto *et al*., 2000; Cocolin *et al*., 2004), which allowed for the discrimination of *Saccharomyces* yeasts species (*cerevisiae, bayanus* and *pastorianus*) and having the resolution ability to differentiate between strains within these species.

In comparison with CHEF karyotyping and interdelta analysis, the multiplexing of microsatellites, GTG_5 and M13, yielded less distinct profiles. The resolution of the technique was limited to identifying single polymorphisms within the profiles indicating possibly a difference between *S. cerevisiae* and *S. bayanus* strains. However, this technique can still be applied as a supportive tool to other molecular techniques.

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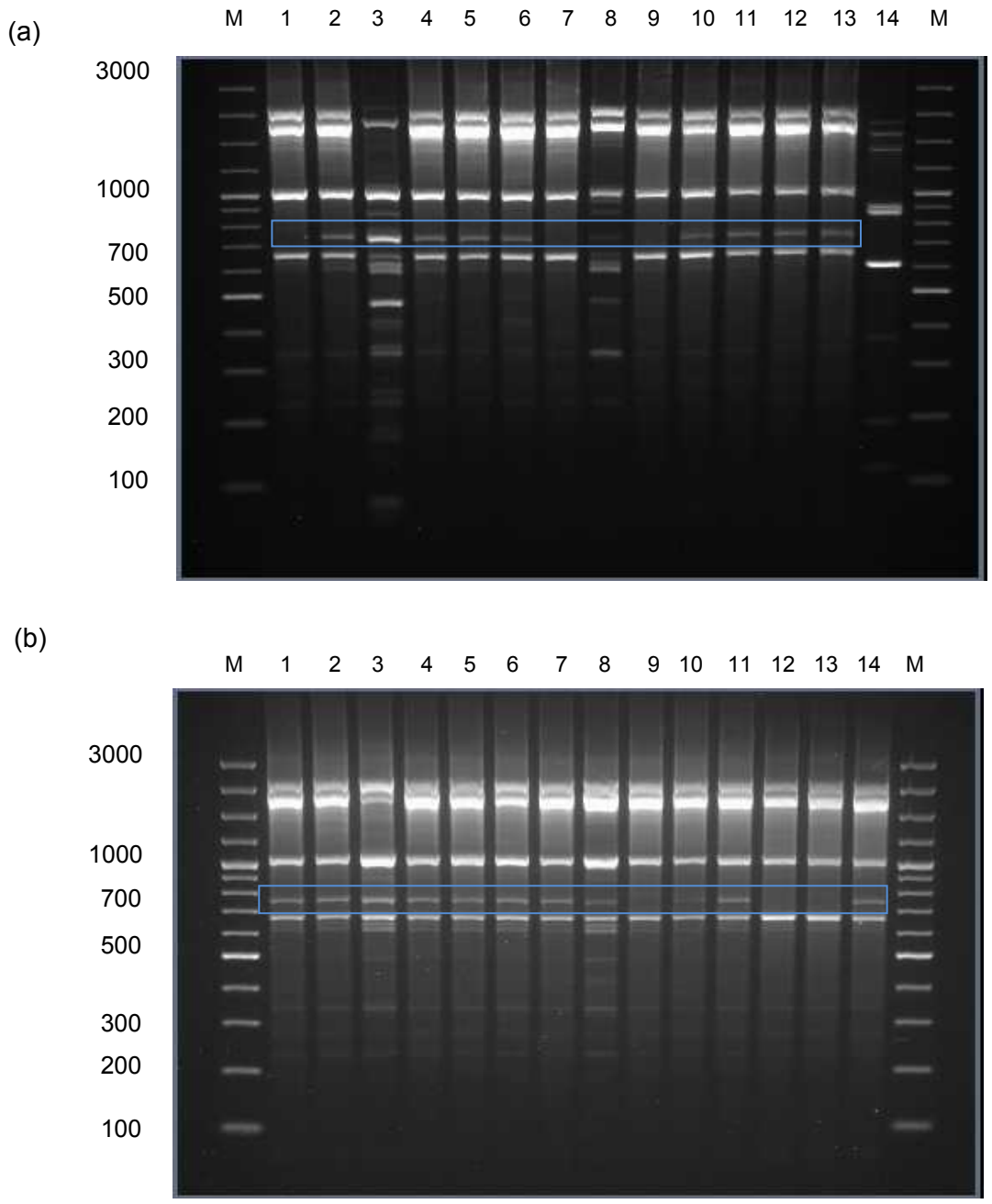

FIGURE 3.6

Products obtained by Multiplexing-PCR microsatellite GTG_5 and minisatellite M13. (a) Marker: Generuler 100 bp Plus DNA marker, lane 1: PDM, lane 2: EC 1118, lane 3: VL3, lane 4: B0 213, lane 5: FX10, lane 6: F15, lane 7: Actiflore PM, lane 8: Merit.ferm, lane 9: Fermol Sauvignon, lane 10: Fermol Chardonnay, lane 11: Arome Plus, lane 12: SUPER16, lane 13: A3B, lane 14: *S. bayanus* reference strain, lane 15: Generuler 100 bp Plus DNA marker. (b) Marker: Generuler 100 bp Plus DNA marker, lane 1: *S. cerevisiae* reference strain, lane 2: IOC 18-2007, lane 3: IOC R9001, lane 4: IOC R9002, lane 5: Fermicru VR5, lane 6: Cabernet Savignon (2881), lane 7: Chardonnay (4882), lane 8: Fermicru 4F9, lane 9: Fermichamp, lane 10: Fermicru LV CB, lane 11: LW05, lane 12: NWS Pinot, lane 13: NWS Chardonnay, lane 14: NWS Merlot, Marker: Generuler 100 bp Plus DNA marker.

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3.3.4 Evaluation of *MET2* gene analysis and species-specific primers

3.3.4.1 *MET2* gene analysis by RFLP

The reference and commercial strains all yielded an amplicon of 580 bp (~600 bp) with no clear distinction (Fig. 3.7, lanes 1-5) Restriction enzyme analysis followed whereby these strains were cleaved with *EcoRI* and *PstI* (Fig. 3.7, lanes 6-10 and lanes 11-15, respectively). Restriction digest of *EcoRI* revealed two fragments for *S. cerevisiae* and only one for *S. bayanus* reference strains. Remarkably most of the commercial wine strains irrespective of oenological designation, resembled the *S. cerevisiae* profile proving that *S. cerevisiae* genomically dominates most commercial strains. However, two strains, AWRI Fusion (Fig. 3.7, lane 9) and Zymaflore X5 (Fig. 3.7, lane 10), differed from the rest and showed three fragments (580 uncut fragment, 369 bp and 211 bp). The restriction digest for these strains were repeated to eliminate the possibility of impurity or cross contamination of the culture was the reason for the patterns. EcoRi digests suggested that these two strains might be hybrids of *S. cerevisiae* and *S. bayanus.* The strain AWRI Fusion (Maurivin) is described as being a hybrid of *S. cerevisiae* and *S. cariocanus*, therefore confirming studies by Masneuf *et al*. (1998) which *MET2* gene analysis could indicate the presence of natural hybrids.

During *PstI* cleavage (Fig. 3.7, lane 11-15), the *S. bayanus* reference strain (lane 12) yielded two fragments (365 bp and 211 bp) and this coincides with the findings of Hansen and Kielland-Brandt (1994) and Naumov *et al*. (1993). All commercial strains, including the two hybrids AWRI Fusion and Zymaflore X5, as well as the *S. cerevisiae* reference strain yielded no cleaved products.

This proved that the two strains mentioned above, were not hybrids of S. *cerevisiae* and *S. bayanus*. However, Masneuf *et al*. (1998) reported that the cider strain CID1 and wine producing strain SU6 yielded three fragments for both *EcoRI* and *PstI.* This was resolved by sequencing and results showed that the *PstI*-uncut *MET2* alleles for these strains were identical matching that of *S. cerevisiae* as well. The *EcoRI*-uncut *MET2* alleles for these strains were also identical and had an 82% homology to that of *S. cerevisiae* and 98% homology to that of *S. bayanus*. A similar reference of analysis should be done to confirm the status of AWRI Fusion and Zymaflore X5*.* Results show that *MET2* gene analysis is a good method for the oenological designation of commercial wine yeast strains from the *S. sensu stricto* complex.

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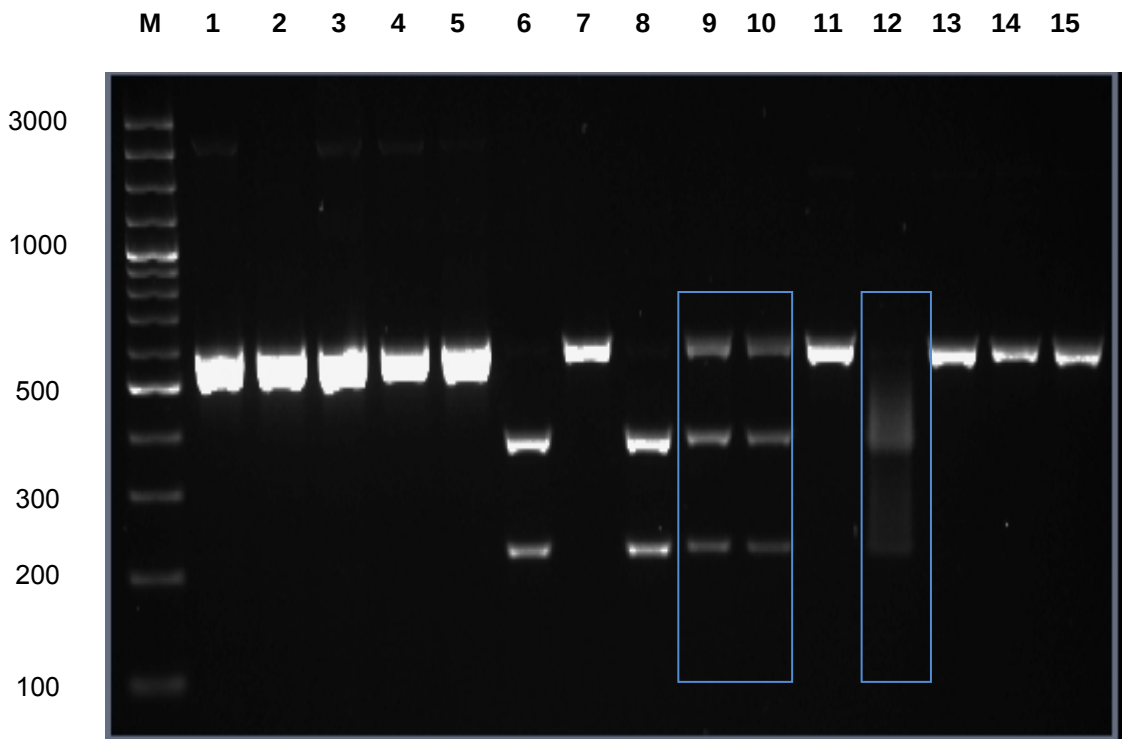

FIGURE 3.7

MET2 gene amplifications (lanes 1 to 5) and restriction fragment length polymorphism (RFLP) products of *EcoRI* (lanes 6 to 10) *and PstI* (lanes 11 to 15) for selected yeast strains. Marker (M): Generuler 100 bp Plus DNA. Lane 1, 6, 11: *S. cerevisiae* reference strain (CBS 1171); lane 2, 7, 12: *S. bayanus* reference strain (CBS 380); lane 3, 8, 13: VIN 13; lane 4, 9, 14- AWRI Fusion; lane 5, 10, 15: Zymaflore X5.

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3.3.4.2 Evaluation of species-specific primers

During this evaluation, four sets of primers (Table 3.2) were tested for the potential to differentiate between two of the seven species in the *Saccharomyces sensu stricto* complex, namely *S. cerevisiae* and *S. bayanus*. These primers were used to evaluate oenological designation of commercial wine yeast strains to support findings of *MET2* RFLP analysis previously discussed.

The SC (*cerevisiae* specific) and SB (*bayanus* specific) primers were tested separately on both reference and commercial wine yeast strains (Fig. 3.8a and b). The primer set SC1/SC2 (Table 3.2) yielded single 1170 bp band for the *S. cerevisiae* reference strain (CBS 1171) and all commercial strains (Fig. 3.8a) as indicated by Josepa *et al*. (2000). A double amplification, 700 bp and 350 bp, was generated in the *S. bayanus* reference strain (CBS 380). The SB1/SB2 primer set yielded an 1170 bp amplicon in the *S. bayanus* reference strain and a number of commercial wine yeast strains, depicted in Fig. 3.8b, lanes 1-4. However, this primer set generated multiple amplicons or non-specific amplifications in the *S. cerevisiae* reference and other commercial wine yeast strains, depicted in Fig. 3.8b. Josepa *et al*. (2000) mentioned similar results in amplifications with SB1/SB2 primer set and that non-specificity disappeared when DNA concentration was lowered. However, when the DNA concentration was lowered inconsistent results were obtained. Amplification with the Torriani *et al*. (2004) YB1f/YB2r primer set generated a single 329 bp band for the *S. bayanus* reference strain (Fig. 3.8c, lane 1). No amplification was visible in the the *S.cerevisiae* reference strain or in any of the commercial wine yeast strains.

The novel *S. bayanus* primer set, SPEOPT18SBAY-F2/R2 (Huang *et al*., 2008), generated a 799 bp amplicon (Fig. 3.9a and b) in the *S. bayanus* reference strain (CBS 380) and multiple bands were generated for the *S. cerevisiae* reference and commercial wine yeast strains (Fig. 3.9a). Amongst these amplicons, noticeably one band was intense and resembled the 1170 bp amplicon generated by the SC1/SC2 and SB1/SB2 primers. Further optimisation of DNA and primer concentrations reduced the multiple bands to none for *S. cerevisiae* reference strain (CBS 1171) and commercial wine yeast strains as indicated in Fig. 3.9b. However, these multiple amplifications or RAPDs, generated for the *S. cerevisiae* reference strain (CBS 1171) and commercial wine strains as in the case of N96 and EC 1118 could be used optionally to differentiate between these or other strains. The commercial strain, Enartis *S. cerevisiae* differed from the other strains and exhibited a second intense 500 bp band (Fig. 3.9a, lane 7). All the species-specific primer sets used in this study showed potential for determining oenological designation of commercial wine yeast strains and supported *MET2* gene RFLP results.

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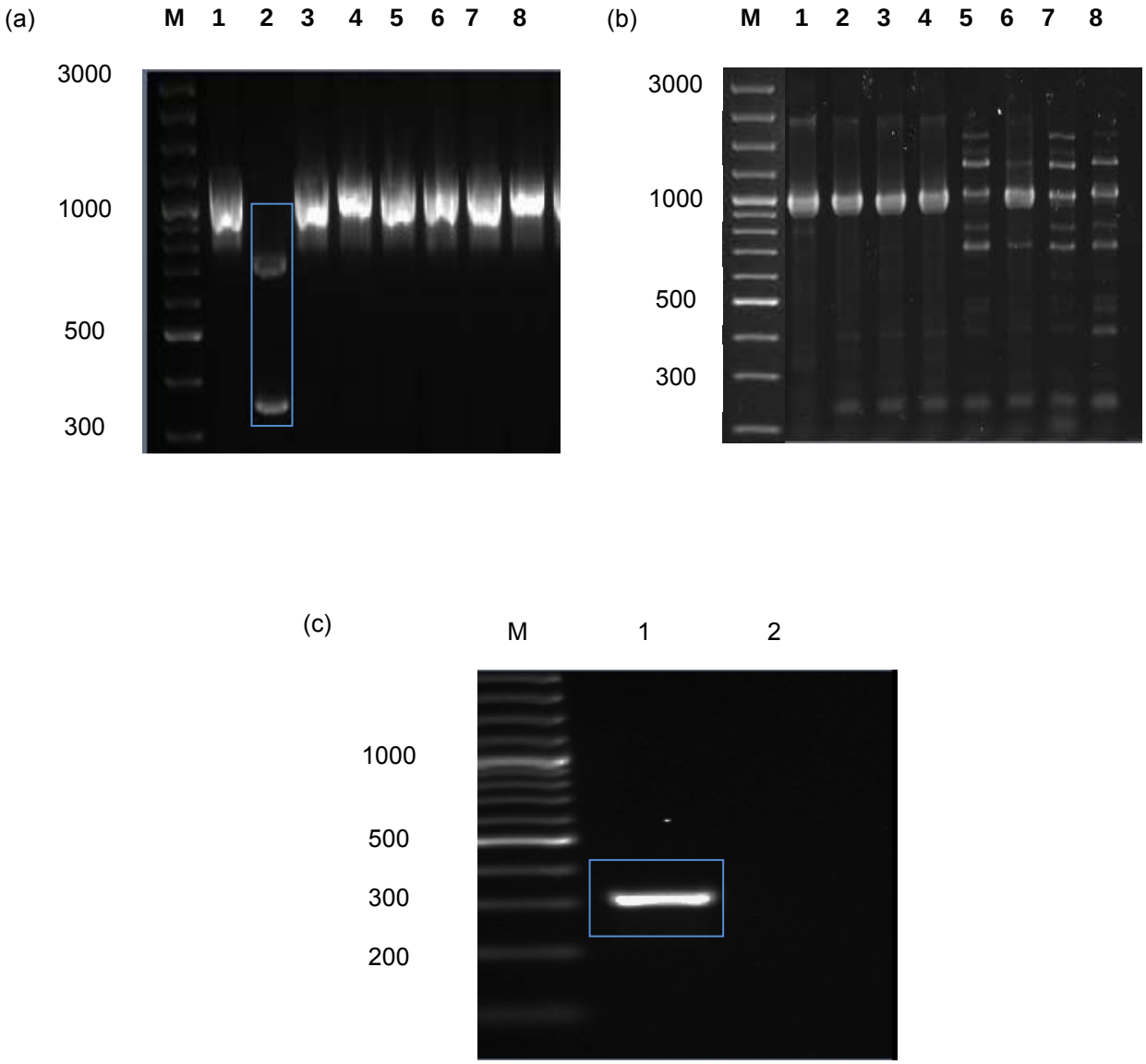

FIGURE 3.8

Species-specific amplications within commercial wine yeast strains in comparison to reference strains of *S. cerevisiae* and *S. bayanus*. (a) Amplification of strains using SC1/SC2 primers in both species (b) Amplification using SB1/SB2 primers in both species. Marker (M), lane 1: *S. bayanus* reference strain, lane 2: N96, lane 3- EC 1118, lane 4: Enartis *S. bayanus*, lane 5: *S. cerevisiae* reference strain, lane 6: VIN 13, lane 7: Enartis *S. cerevisiae*, lane 8: Fermol Chardonnay. (c) Illustrates the amplification using the YB1f/YB2r *S. bayanus* specific primer set.

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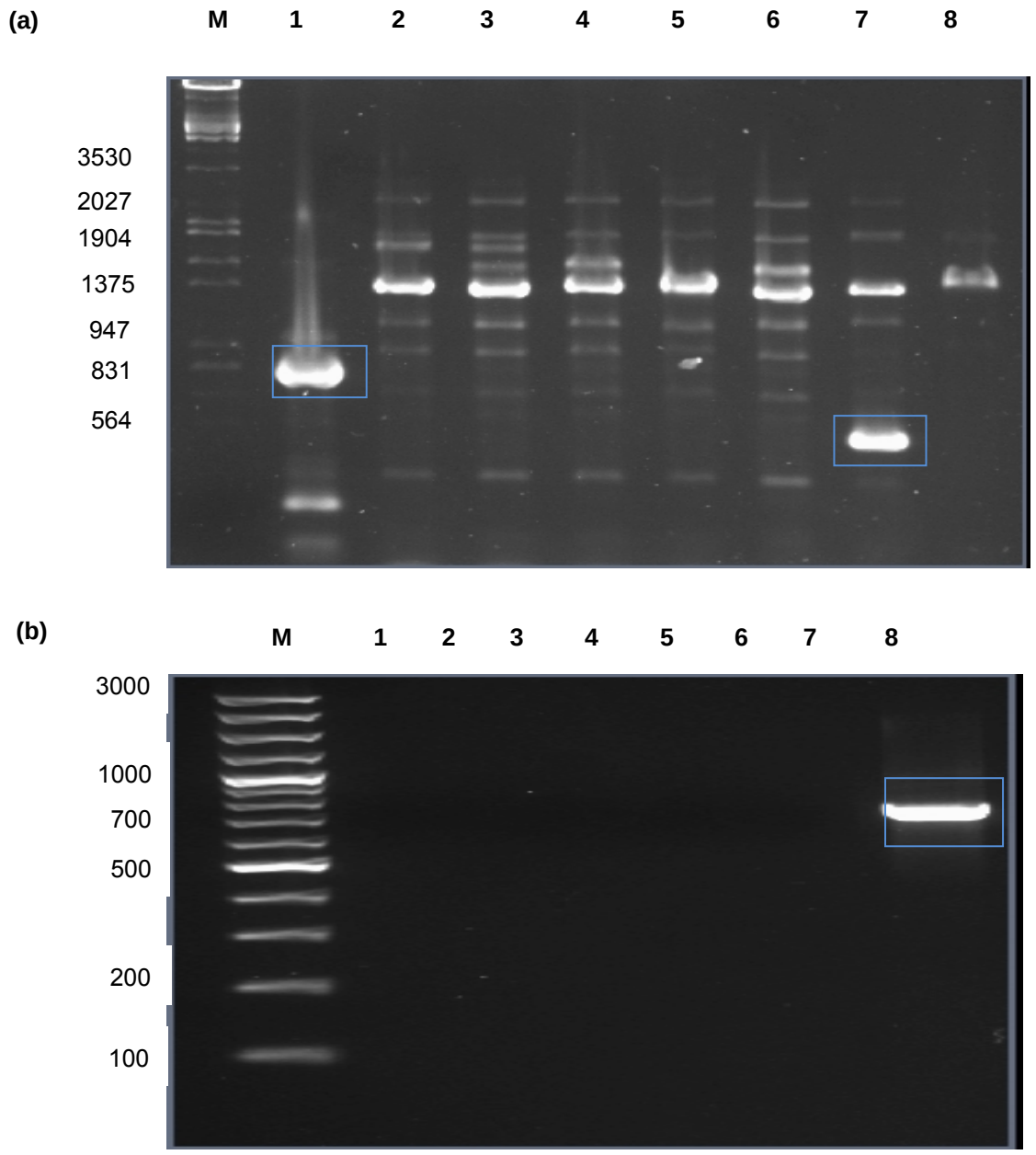

FIGURE 3.9

Amplification reactions with the SPEOPT18Sbay-F2/R2 *S. bayanus* species-specific primers for *Saccharomyces* reference and commercial wine yeast strains. (a) over amplification using this specific primer set, Marker- λDNA *EcoRI/HindIII*, lane 1: *S. bayanus* reference strain, lane 2: N96, lane 3: EC 1118, lane 4: Enartis *S. bayanus*, lane 5: *S. cerevisiae* reference strain, lane 6: VIN 13, lane 7: Enartis *S. cerevisiae*, lane 8: Fermol Chardonnay. (b) Illustrates the the reduced DNA and primer concentrations with a single amplicon for the *S. bayanus* reference strain (lane 8).

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3.3.5 Creation and evaluation of database

Profiles generated by CHEF karyotyping and the interdelta PCR amplifications (delta1-2 and delta12-21) were used to create a molecular fingerprint database with FPQuest ™ software. A basic view of the yeast database containing CHEF and interdelta fingerprints are shown in Fig. 3.10. Optimisation of the database included, evaluation of different gel electrophoretic systems, agarose gel concentrations, as well as different correlation coefficients such as Pearson or Dice. The accuracy and efficiency was tested using the CHEF karyotyping, because it was the primary method and showed higher resolution amongst commercial wine yeast strains.

Two commercial yeasts, NT 45 and EC 1118, were selected as test organisms (Table 3.3) and were treated as unknown strains. The standard procedures were followed for CHEF karyotyping and the unknown strains were then compared to all 64 yeasts in the database. The test organism, Unknown 2, correctly clustered with NT 45 (Fig. 3.11). The test organism, Unknown 1, clustered with EC 1118 and Fermicru 4F9. In this case, the banding pattern was slightly different compared to the commercial EC 1118 strain that was already in the database. Small fluctuations in the agarose gel concentration due to water loss as a result of heating during preparation can alter structural composition of the agar. This could explain the difference in the spatial separation of the chromosomes. Cluster analysis is based on pairwise grouping of strains. The addition of more strains to the database affects the similarity and cophenetic correlation, as well as the clustering. Each new yeast entry will therefor affect the clustering of the yeast strains. However, clustering of test organisms with their respective commercial strains in Fig. 3.11 provides evidence that the database is fully functional and can be used for identification of unknown yeast strains and comparative studies.

The functionality of the databse was furthermore tested in two independent case studies. In case study 1, seven commercial wine yeast strains was submitted as unknown isolates from the University of KwaZulu-Natal. Using the yeast database these isolates were successful identified (data not shown). In case study 2, commercial spoiled bag-in-boxed wine was investigated. This was a confidential service for a client. A yeast strain was isolated from the the spoiled wine and compared to commercial yeast strains used by the boxed wine producer. The isolated yeast differed from strains normally used by the producer (data not shown). The isolated yeast was also compared to the remainder of commercial yeast strains in the database (data not shown). The isolated yeast strain did not correspond to any of the strains in the database, which indicates that the spoilage is probably a natural isolate or commercial yeast that has not yet been included in the database.

With a planned expansion of the database to include more commercial strains, future identification of unknown yeast isolates will be more successful. The database will also be valuable during future comparative studies.

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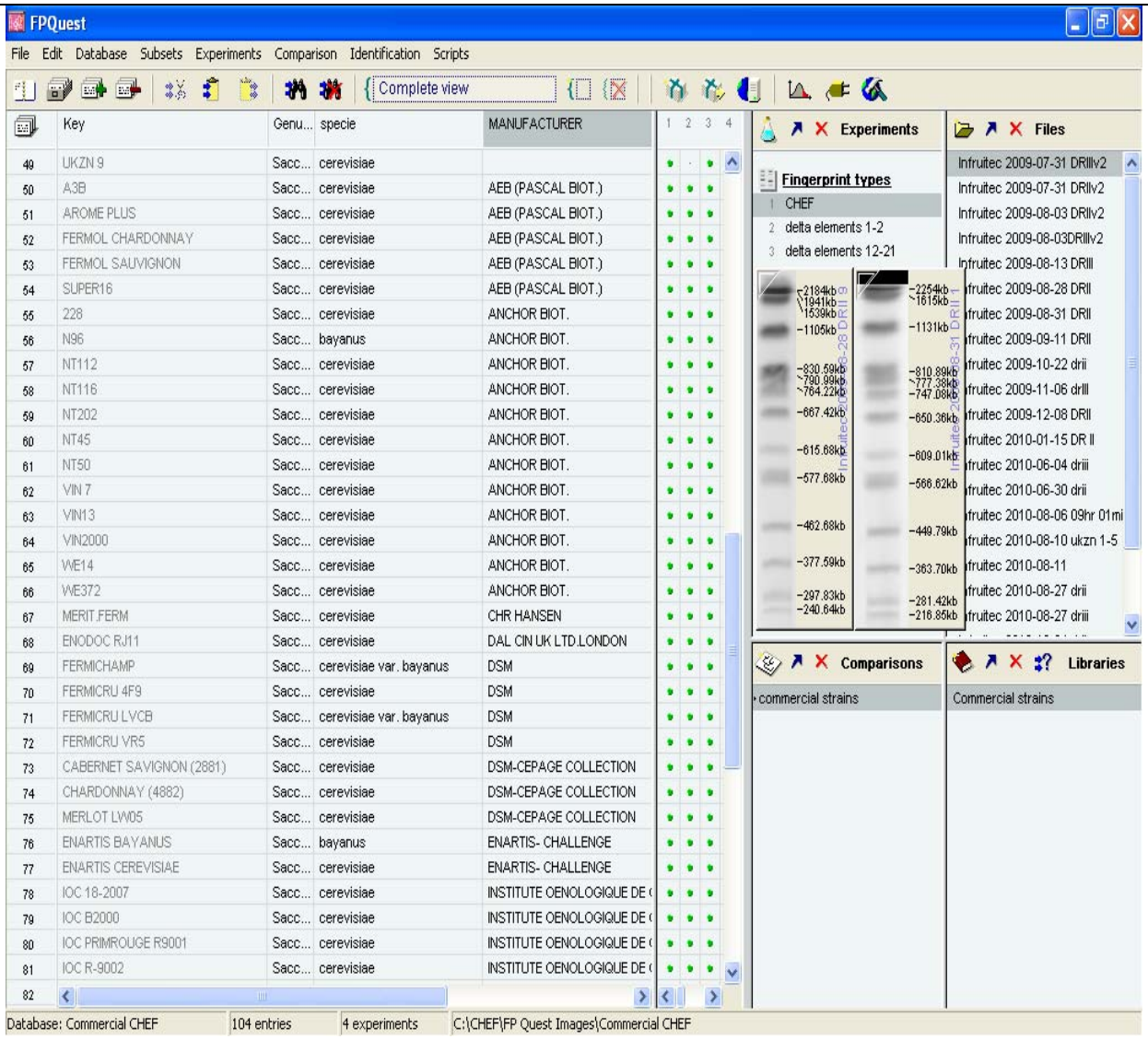

FIGURE 3.10

A basic view of the yeast database containing CHEF and interdelta fingerprints for identification and comparison of unknown yeasts as well as commercial wine yeast strains.

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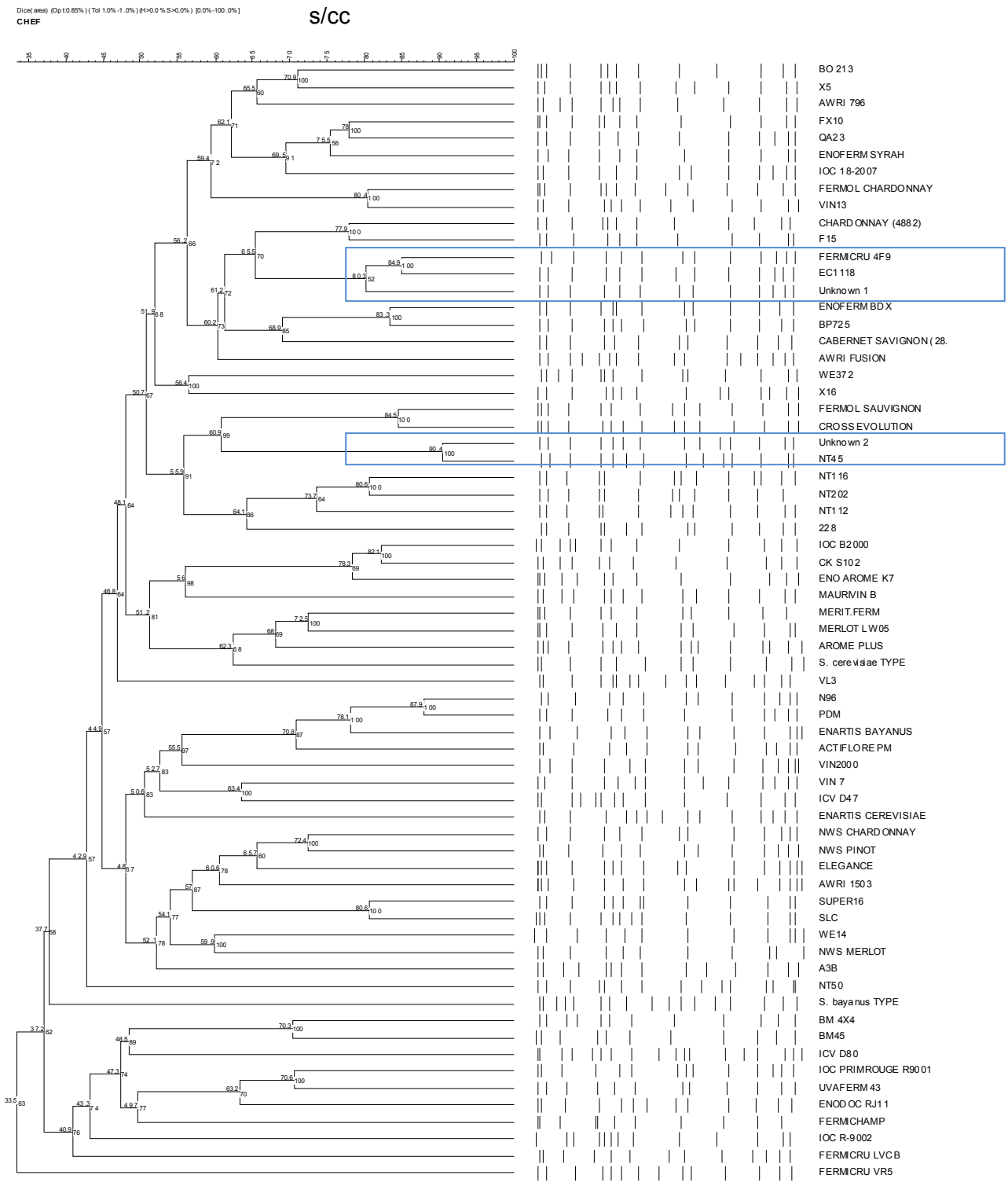

FIGURE 3.11

Dendogram showing the clustering of test organsims (TO) 64 yeast strains obtained by numerical analysis of CHEF karyotypes. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Similarity (s) and cophenetic correlations (cc) between strains were calculated based on the Dice coefficient.

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3.4 CONCLUDING REMARKS

CHEF karyotyping is still widely used due to its powerful resolution of the *Saccharomyces sensu stricto* complex group, as well as strains within species. The potential of the methodology was confirmed during this investigation, as it was possible to differentiate between the 62 commercial wine yeast strains. However, this technique is still time-consuming and relatively costly. The results in this study showed that (PCR) methods can alternatively be used and systematically applied as supportive tools to CHEF karyotyping. Interdelta PCR was very useful, but CHEF karyotyping proved to better for differention of wine yeast strains. However, interdelta amplification is less time-consuming and the results are obtained a lot faster. It was further showed that PCR methods aimed at identifying species can be useful in identifying unknown strains belonging to specific oenological species.

*MET*2 gene RFLP and species-specific primers successfully designated commercial wine yeast as *S. cerevisiae. MET*2 gene RFLP showed great resolve with the identification of two strains, Zymaflore X5 and AWRI Fusion, as hybrid strains. During the evaluation of these typing techniques a database was created, containing molecular profiles for CHEF karyotyping and interdelta regions. Clustering of test organisms, NT 45 and EC 1118, with their respective commercial counterparts in the database as well as case studies provides evidence of the accuracy and functionality of the database. The database can therefore be used to type unknown isolates or test strain authenticity. The database will also allow for comparisons of numerous yeasts without running many agarose gels. Future work would include evaluating various others molecular methods and adding those worthy fingerprint types to the database. The expansion of the database will include the incorporation of non-*Saccharomyces* wine yeasts and molecular techniques that are useful in discriminating amongst these wild yeasts.

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Chapter 4

General discussion and conclusions

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GENERAL DISCUSSION AND CONCLUSIONS

4.1 DISCUSSION AND CONCLUSION

In chapter 2, current and past technologies that has been useful for the identification, characterisation and in some cases classification of wine yeasts were discussed. When it comes to wine it is necessary to know which organisms play a crucial role during the fermentation process. The plating out of yeasts, *Saccharomyces* or non-*Saccharomyces* on differential media and the use of API systems are still general practise in modern day laboratories and are seen as the stepping stones of investigation. However, molecular techniques now provide us with the potential to identify yeasts not just at genus or species level, but have also given us resolution for strain specificity. Modern day molecular techniques also allow for the detection of previously non-culturable yeast species directly from the sample, whether it is wine or grape juice. In the past, these species would have gone undetected by previous screening methods. Molecular techniques present a faster method of analysis, which is very important when it comes to the wine industry with regard to time and financial losses.

Chapter 3 described the evaluation of molecular techniques and their ability to differentiate between 64 yeast strains. The use of CHEF karyotyping and PCR of interdelta regions to setup a wine yeast database was also discussed. These yeast cultures included two reference strains, *S. cerevisiae* (CBS 1171) and *S. bayanus* (CBS 380) and 62 commercially available wine yeast strains, designated either as *S. cerevisiae* or *S. bayanus*, as well as some hybrids. CHEF karyotyping was used as the platform technique, as it is often used for identification and characterisation of wine yeasts. CHEF has in shown great resolution when it comes to the differentiation of wine yeast strains because these strains show a high level of chromosome polymorphisms (Rachidi *et al*., 1999). Many authors have used CHEF to differentiate and also determine genetic relatedness amongst yeast species with in the *Saccharomyces sensu stricto* group. During this study, 64 strains were evaluated and all were successfully differentiated by CHEF karyotyping. The CHEF karyotypes of the commercial strains when compared to the reference strains confirmed their status as hybrids. Industrially these wine yeasts are cross bred amongst several species within the *Saccharomyces sensu stricto group*. It has been suggested that, that because they are aneuploidy or polyploidy, commercial strains are well adapted for anthropic environments such as wine fermentations (Salmon, 1997, Querol *et al*., 2003). A dendogrammic illustration for CHEF karyotypes could not provide any evidence of geographical relationships amongst strains. However, there are indications of phylogenetic correlations between commercial strains from some of the manufacturers. This is expected as some manufacturers have natural isolates that are used in breeding programmes.

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Alternatively to CHEF, three PCR methods, interdelta priming, micro- and minisatellites and species-specific priming, were used as these method showed promise during initial screenings. Optimisation of PCR conditions included evaluating different genetic source materials (Genomic DNA and colony-PCR), DNA concentrations and optimal hybridisation temperatures of primers. Genomic DNA provided more dependable results, whereas colony-PCR was often inconsistent. These inconsistencies normally occur because of impurities that interfere with the PCR reaction. However, the two sources delivered relative similar results.

Delta elements are subtelomeric repeated sequences and these sequences are described as being responsible for the chromosomal polymorphisms, which links this PCR method to CHEF karyotypes. However, the number and location of these sequences themselves are intraspecific which make them excellent fingerprinting mechanisms. Optimisation of delta amplifications is always critical, as too high DNA concentrations and low hybridisation temperatures generate ghost bandings patterns. During this study, delta primers combinations, 1-12 and 12-21 were used. For this PCR method, optimisation of PCR reaction was critical. Optimal conditions were found with 20 ng DNA and a primer hybridisation temperature of 48°C. These conditions varied considerably from what other studies had indicated. Reproducibility within labs may vary because of these factors. Compared to CHEF, only 59 and 62 distinct electrophoretic profiles were distinguished. The bands generated during amplification ranged from 100 to 3000 bp, considerably smaller in size compared to CHEF karyotyping. The delta12-21 primer combination generated more bands compared to delta1-2, as suggested by Legras and Karst, (2003). The two type strains, *S. cerevisiae* and *S. bayanus*, as with CHEF karyotyping, yielded distinct profiles using interdelta regions. However, it was evident that the profiles of the commercial strain had once again a hybrid nature. Distinct bands from both type strains were found in all commercial strains. These hybrid profiles also illustrated bands not found in the profiles of the type strains, which indicates genetic information from other *Saccharomyces* species. Compared to CHEF karyotyping interdelta regions provided a faster method of analysis, with a good resolution at strain level, however less than CHEF. Comparison of the techniques also suggested phylogenetic relatedness amongst commercial wine yeast strains.

Amplification of micro- and minisatellite sequences, GTG_5 and M13, were used in a multiplex-PCR reaction to maximise the possible potential to differentiate between the 64 yeast strains in this study. These primers are generally characterised as having high hybridisation temperatures (55-66°C). Multiplexing using these microsatellites primers did not have the same discriminatory power of CHEF karyotyping interdelta primers. The only noteworthy result was the amplification of a 730 bp amplicon. This amplicon in turn was used to align specific commercial strains with the *S. cerevisiae* type strain (CBS 1171) or *S. bayanus* type strain (CBS 380). However, future work should investigate satellite primer sets. Currently, specific microsatellite loci, which are very hypervariable, are found in databases with the genotypes of

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several yeast strains, include that of some commercial wine yeasts. The use of M13 bacteriophage sequence has also led to other bacterial sequences such as, enterobacterial repetitive intergenic consensus elements and extagenic palindromic elements being explored in the identification and differentiation of yeasts strains.

To determine oenological designation of commercial wine yeast strains, *MET2* gene RFLP analysis and species-specific primers were investigated. *MET2* gene analysis has in the past been used to determine or evaluate the oenological designation of *Saccharomyces* yeasts. This study showed that 60 of the commercial strains used, resemble the *S. cerevisiae* types strain. However, none of the commercial strains bore a resemblance to the *S. bayanus* profile. However, two strains did show a hybrid profile of both type strains when digested with *EcoRI*. These hybrids *MET2* genes were also digested with *PstI*, but did not match the profile obtained for the *S. bayanus* type strain. This was an indication of their hybrid nature with other *Saccharomyces* species. To verify these findings, four sets of species-specific primers were used. The *S. cerevisiae* specific primers (SC1/SC2; Josepa *et al*., 2000) indicated that all commercial strains used during the study was *S. cerevisiae* of origin and were completely different to the *S. bayanus* type strain. Initially, mixed results were obtained with the *S. bayanus* specific primers (SB1/SB2; Josepa *et al*., 2000). Several commercial strains showed amplification similar to that of *S. bayanus* type strain. However, multiple amplifications were also visible for other strains, but with a reduction in DNA concentration these multiple bands disappeared.

To confirm previous results, two *S. bayanus* specific primer pairs (YB1f/YB2r (Torriani *et al.*, 2004); SPEOPT18 f2/SPEOPT18 r2 (Huang *et al*., 2008)) were investigated as well. As with the previous pair, multiple DNA concentrations were analysed. Single amplifications with both primer sets in the *S. bayanus* type strain confirmed that all of the commercial strains are S. cerevisiae in origin. However, species-specific priming also show that genetic material from both types strain maybe scattered throughout these commercial strains genomes. During initial amplifications multiple bands were obtained with a higher DNA concentration in all strains. This amplification also provided a possible application whereby it could be use for strain differentiation.

The last part of chapter three discusses and analysis the establishment of a database with various molecular libraries using FPQuest™ software. CHEF karyotyping was used to create the first library and subsequently used as an example for testing and evaluating the database. To evaluate accuracy and effiency, commercial strains, EC 1118 (unknown 1) and NT 45 (unknown 2), were used as test organisms by setting up a UPGMA dendogram based on a Dice similarity coefficient. The Dice coefficient was selected based on past studies and literature on similar work. Other coefficients such Pearson was evaluated, but gave inconsistent results (data not shown). Clustering of test organisms with matching commercial yeast strains indicated that the database is accurate and functional. However, optimisation within the

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software is still needed to find the optimal settings. Subsequently, two case studies were used to test functionality of the database. In case study 1, seven commercial wine yeast strains were received as unknown isolates from the University of KwaZulu-Natal and were successfully identified. Case study 2 presented a different assessment, whereby an unknown yeast strain was isolated from a spoiled boxed wine (confidential report). Results indicated that the isolate did not match any of the commercial strains used by the wine producer or strains within the database. The results of above mentioned studies showed great accuracy, but also highlighted concerns with regard to agarose gel concentration, the use of different electrophoretic separation equipment and the need to incorporate more commercial wine yeast strains in the database. These are aspects that in future will be addressed.

4.2 INDUSTRIAL IMPORTANCE AND FUTURE PROSPECTS

The establishment of a database containing CHEF and interdelta molecular profiles will serve as a tool for yeast profiling studies. The database will make it easier to compare and identify unidentified yeasts from vineyards and genebank culture collections. The database will also facilitate comparative studies on commercial wine yeasts and the spread of commercial strains to vineyards. Furthermore a quality control service will be available to yeast manufacturers and/or industrial companies, whereby genetic drift of commercial strains can be monitored as well as the detection of spoilage yeasts.

Future prospects include the expansion of the database, which will include sourcing more commercial wine yeast strains and newer technologies for differentiation of strains. A non-*Saccharomyces* yeast and bacteria database is also envisioned.

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